“STUDIES ON PREVALENCE, PATHOLOGY AND MOLECULAR DIAGNOSIS OF INFECTIOUS BURSAL DISEASE IN BROILERS”

M. V. Sc. THESIS

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VITA

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CERTIFICATE – I

This is to certify that the thesis entitled "**Studies on Prevalence, Pathology and Molecular Diagnosis of Infectious Bursal Disease in Broilers**" submitted in partial fulfillment of the requirements for the Degree of **Master of Veterinary Science** of the Chhattisgarh Kamdhenu Vishwavidyalaya, Durg (C.G.) is a record of the bonafide research work carried out by **Lokesh Kumar Mohabe** under my guidance and supervision. The subject of the thesis has been approved by the student's Advisory Committee and the Director of Instructions.

No part of the thesis has been submitted for any other degree or diploma or has been published/published part has been fully acknowledged. All the assistance and help received during the course of investigations have been duly acknowledged by him.

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CERTIFICATE – II

This is to certify that the thesis entitled "**Studies on Prevalence, Pathology and Molecular Diagnosis of Infectious Bursal Disease in Broilers**" submitted by **Lokesh Kumar Mohabe** to the Chhattisgarh Kamdhenu Vishwavidyalaya, Durg (C.G.) in partial fulfillment of the requirements for the Degree of **Master of Veterinary Science** in the Department of Veterinary Pathology has been approved by the student's Advisory Committee after oral examination in collaboration with the External Examiner.

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Date: *LOKESH KUMAR MOHABE*

Place:

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Abbreviation		Full Form
%	-	per cent
@	-	at the rate of
µg	-	microgram
µl	-	microlitre
BF	-	bursa of Fabricius
Bp	-	base pair
DCT	-	distal convoluted tubules
DLC	-	differential leucocyte count
ELISA	-	enzyme linked immune sorbent assay
g/dl	-	gram per deciliter
H&E	-	haematoxylin and eosin
Hb	-	haemoglobin
hr	-	hour(s)
i.e.	-	that is
IBD	-	infectious bursal disease
IBDV	-	infectious bursal disease virus
L	-	litre
mg	-	milligram
PCT	-	proximal convoluted tubules
PCV	-	packed cell volume
RT-PCR	-	reverse transcriptase polymerase chain reaction
TEC	-	total erythrocyte count
TLC	-	total leucocyte count
VVIBD	-	very virulent strain of IBDV

CHAPTER-I

Introduction

CHAPTER – I

INTRODUCTION

The production and consumption of eggs and poultry meat have been increasing worldwide over the last three decades as the consumption of eggs has doubled and that of chicken meat has tripled (Jordan and Pattison, 2001).

With increasing industrialization and intensification of rearing systems, the disease pattern in fowl is changing. In developing countries, the infectious diseases still play a predominant role in deciding the economic returns from the poultry industry. According to Sainsbury (1992), tropical countries have more problems with infectious diseases due to the climatic circumstances.

Infectious bursal disease (IBD) was discovered in 1957 in Gumboro, Delaware, USA. As a result, the disease is often referred to as Gumboro. Not long after IBD was first reported, it was being recognized in poultry populations throughout the world.

Infectious bursal disease (IBD) is a highly contagious disease of young chickens caused by Infectious bursal disease virus (IBDV), characterized by immunosuppression and mortality generally at 3-6 weeks of age. It has contributed significantly in overall losses to poultry industry because of increased mortality due to IBD and other diseases occurring as a result of vaccination failures due to immunosuppressive effect of the disease. The reemergence of IBD particularly in last couple of decades in variant or highly virulent forms has been the cause of significant economic losses. In India, IBD was first recognized in Uttar Pradesh in 1971 (Mohanty *et al.*, 1971) followed by virus isolation by Jayaramiah and Mallick (1974). Ever since the first outbreak of IBD in India, the disease has remained a major threat to poultry industry in almost all the

states. However, the devastating effect was realized only when severe outbreaks occurred in Maharashtra (Ajinkya *et al*., 1980) and Andhra Pradesh (Verma *et al*., 1981).

IBDV is a small, non-enveloped virus, belonging to genus *Avibirnavirus* of the family *Birnavirdae,* which is characterized by a bisegmented double stranded RNA genome (Kibenge *et al*., 1988). Two recognized serotypes of IBDV are established. Serotype 1 includes pathogenic viruses capable of causing disease in chickens. Serotype 2 is pathogenic to turkey and so far, its pathogenecity to chickens has not been described. Atleast six antigenic subtypes of IBDV serotype 1 have been identified by *in vitro* cross neutralization assay (Jackwood and Saif, 1987). Viruses belonging to one of these antigenic subtypes are commonly known as variants, which were reported to break through high levels of maternal antibodies in commercial flocks causing up to 60 to 70 per cent mortality rate in chicken (Eterradossi *et al*., 1992). It is economically important to the poultry industry worldwide due to increased susceptibility to other diseases and negative interference with effective vaccination. In recent years, vvIBDV causing severe mortality in chicken have emerged in Europe, Latin America, South - East Asia, Africa and the Middle East.

The VP2 protein is the major protective antigen of IBDV that contains specific epitopes responsible for inducing neutralizing antibody responses. Amino acid changes that occur within the variable region of VP2 can lead to variations in antibody recognition, as well as, antigenicity, immunogenicity, virulence, and tissue tropism of IBDV strains. There is a hypervariable region of the VP2 gene that contains most of the differences between IBDV strains. Since this hypervariable region of the VP2 gene exhibits a number of changes depending on the IBDV strain, most of the molecular techniques for genotyping IBDV are focused on analyzing this region.

When IBD was first recognized, it was characterized by whitish or watery diarrhea, anorexia, depression, trembling, weakness, and death. This clinical IBD was generally seen in birds between three and eight weeks of age. Infected chickens with classical or very virulent strains of IBDV may show diarrhea, muscular haemorrhage and necrosis of the bursa of Fabricius. Some birds with minor bursal lesions can be found dead, while others survive despite extensive bursal damage. The course of the disease runs approximately 10 days. Mortality usually ranges from 0 - 30 percent. Field reports suggest leghorns to be more susceptible to IBD.

Subclinical IBD was later recognized and is a greater problem in commercial poultry than the clinical disease. It is generally seen in birds less than three weeks of age. No clinical signs are generally seen. This early infection results in a lymphoid depletion of the bursa of Fabricius. The bird is immunologically crippled and unable to fully respond to vaccine or field virus. In addition, the bird may be susceptible to agents that are not normally pathogenic (adenovirus, clostridial infections).

Detection of IBDV in clinical samples is conveniently carried out serologically by AGPT and ELISA using hyperimmune antiserum (Nachimuthu *et al.*,1995), which besides being less sensitive and cumbersome respectively, are often difficult to interpret. The nucleic acid based detection tests like RT-PCR and nucleic acid hybridization, overcome these problems and therefore have been used for the detection and differentiation of various IBD viruses (Lin *et al.*, 1994 and Kataria *et al.*, 2000). Currently, RT-PCR is the molecular tool frequently applied for IBD diagnosis (Muller *et al.*, 2003).

At present there is no baseline data on the prevalence of IBD in broilers in Chhattisgarh. Moreover, only traditional methods of diagnosis e.g., AGPT and

histopathology are being used at present for diagnosis of IBD. So there is a need for molecular diagnosis of IBD for prevention and control of IBD.

OBJECTIVES

Keeping the above points in view, the present research work will be executed with the following objectives:

1. To study the prevalence of IBD in broiler birds in Durg, Rajnandgaon and Raipur district of Chhattisgarh in the poultry farm under study.
2. To study the haematological alteration in the IBD affected birds.
3. Standardization of PCR for detection of IBD virus from clinical samples of poultry showing clinical signs and lesions of IBD.
4. To study pathomorphological alterations in IBD affected birds.
5. To assess the antibody titer of IBD infected flock by ELISA test.

CHAPTER-II

Review of Literature

CHAPTER - II

REVIEW OF LITERATURE

2.1 History

The IBDV was first recognized by Cosgrove in 1962, because of the kidney damage found in birds that succumbed to infection. The disease was referred to as “avian nephrosis” (Lukert and Saif, 2004). Winterfield and his coworkers isolated an agent in embryonated eggs and was referred to as “infectious bursal agent” and identified as the cause of IBD (Lukert and Saif, 2004). Due to the pathogonomic lesions observed in the bursa of Fabricius (BF), Hitchner proposed the name “infectious bursal disease” (Lukert and Saif, 2004).

In 1980, the existence of a second serotype was reported (McFerran *et al*., 1980). In serotype I, six subtypes were defined on the basis of neutralization test, of these first five were called standard because vaccines from any of these isolates protected against virulent viruses in these subtypes. In contrast, virulent virus isolates in subtype 6 were called variant, because they were not well protected against by standard vaccines (Rosenberger and Cloud, 1986).

The control of IBD was complicated by the recognition of “variant” strains of serotype 1 IBDV, which were first found in the Delmarva poultry producing area of the USA (Lukert and Saif, 2004). In the late 1980s, a very virulent strain of IBDV (vvIBDV) was isolated in the Netherlands, and quickly spread to Africa, Asia and lately to South America. The vvIBDV has not been reported from Australia and

New Zealand (Lukert and Saif, 2004). but recently vvIBDV outbreaks in layer flocks were reported in United States (Jackwood *et al.*, 2009).

2.2 Prevalence of IBDV

IBD was first recognized as a disease entity in "Gumboro" district of Delaware State in USA in 1957 by Cosgrove (1962). In India, the disease was first noticed in 1971 (Mohanty, 1971). Sporadic outbreaks of IBD were seen in Karachi and Faisalabad areas in 1983 (Anjum *et al*., 1993). They reported that non-vaccinated flocks (56.58 %) suffered more than the vaccinated flocks (43.42 %).

In 1985, variant IBDV strains emerged in USA (Rosenberger *et al.*, 1985) which did not induce overt clinical changes typical of classic IBDV. Instead, prolonged and repeated respiratory problems and poor performance followed early bursal atrophy. Vaccines developed for control of classical IBDV were not effective and hence, variant vaccines were introduced (Snyder *et al.*, 1992). In 1987/88, vvIBDV strains capable of causing 30 to 70 per cent mortalities in broilers and layers were isolated in Holland, Belgium and UK (Van den Berg and Meulemans, 1991). Since then, outbreaks of vvIBDV have occurred in most European countries as well as Africa, Japan, China and South East Asia. vvIBDV were able to break through the maternal as well as active immunity induced mainly by classical or mild IBDV vaccines.

Zahoor (2010) determined the prevalence of IBDV in Pakistan commercial broiler farm both in vaccinated and non-vaccinated birds of different age groups. He observed that the percentage of positive results in all age groups despite of the discrimination between vaccinated and non-vaccinated birds was 26.53 % in 0-3 weeks, 56.30 % in 3-6 weeks and 33.34 % in 6-8 weeks of age.

Kim *et al*. (2010) revealed the presence of four genotypes of IBDV such as very virulent, classical virulent, antigenic variant and intermediate vaccine strain in South-Korea and these IBDV strains were discriminated using genetic characterization of virion protein 2 gene for efficient diagnosis and disease control.

Jayaramiah and Mallick (1975) conducted a serological survey of IBD in chickens which revealed the prevalence of precipitating antibodies against IBDV to be 22.14 per cent in Andhra Pradesh, 9.09 per cent in Tamil Nadu, 32.00 per cent in Uttar Pradesh and 30.00 per cent in Karnataka.

Dongaonkar and Rao (1979) detected the precipitating antibodies against IBDV antigen from the sera samples of the poultry, and found 17.03 per cent prevalence in Madhya Pradesh and 13.19 per cent in Maharashtra State.

Gill *et al.* (1988) reported IBD outbreak from Punjab, during which 3000 chicks died out of 7800 chicks on the farm, within a week.

Balachandran *et al*. (1991) diagnosed and reported IBD in layer flock of White Leghorn birds aged about 11 weeks from various parts of Tamil Nadu State.

Nachimuthu *et al.* (1993) also reported that the prevalence of IBDV in Tamil Nadu based on isolation and identification of the virus, with simultaneous screening for the possible presence of the variant type of IBDV.

Sah *et al.* (1995) investigated with the emergence of vvIBDV, the disease was reported from all parts of the country with alarming consequences. With each outbreak, there was emergence of more virulent IBDV than the previous one. The authors reported outbreaks of acute IBD characterized by high mortality rate in broiler flocks in Northern India followed by similar episodes in rest of the country

throughout the year 1993-94. The mortality ranged from 10 to 75 per cent in 3 to 16 weeks old White Leghorn and 10 to 40 per cent in broiler flocks of 72 poultry farms in different states of the country.

Sivaseelan and Balachandran (2003) studied fifteen outbreaks of IBD and concluded that there was a pathogenic shift in serotype 1 of IBDV due to extreme vaccination pressure, which ultimately resulted in more virulence and high mortality up to 50 per cent in Namakkal area of Tamil Nadu.

Jaisankar *et al.* (2003) investigated the pattern of viral diseases of poultry in Namakkal for the period of 10 years. They found that the disease was more prevalent in birds of 0 - 5 weeks age group (94.06%) and highest mortality percentage was due to IBD (69.18%), with the maximum incidence in winter (43.93%) and summer (39.74%) followed by monsoon (16.74%).

2.3 Virus characteristics

2.3.1 Structure and properties of IBDV

IBDV is a small, non-enveloped virus of diameter 60 nm. It is a double-stranded RNA (dsRNA) virus with a bisegmented genome, and it belongs to the genus *Avibirnavirus* of the family Birnaviridae (Dobos *et al*., 1979). The virion has a single capsid shell of icosahedral symmetry composed of 32 capsomeres (Hirai and Shikamura, 1974). The virus is resistant to ether and chloroform and it is inactivated at a pH of 12, but unaffected at pH 2.0. The infectivity of the virus is significantly reduced by exposure to 0.5 percent formalin for 6 hrs (Benton *et al*., 1967). The virus is more resistant to heat, UV radiation and photodynamic inactivation when compared to chemical method of inactivation (Petek *et al*., 1973).

The virus is highly stable and has a tendency to persist in the environment despite thorough cleaning and disinfections (Kibenge *et al*., 1988). The virus was found to remain viable for up to 60 days in poultry house litter (Vindevogel *et al*., 1976).

2.3.2 IBD Viral proteins

The virus is reported to have at least four structural proteins (Hirai *et al*., 1979) and as many as seven structural proteins (Nick *et al*., 1976). The nucleic acid and structural proteins of IBDV serotype I were compared with those of serotype II. The growth curves of both the serotypes were similar but the latent period and virus yield were different. There were differences in the molecular weights of viral proteins VP3 and VP4 from the two serotypes (Jackwood *et al*., 1984).

VP1- the RNA dependent RNA polymerase (RdRp) of the virus is present in the virion both as a free polyprotein and as a genome linked protein (Muller and Nitschke, 1987 and Kibenge and Dhama, 1997). It also plays a key role in the encapsidation of the viral particles (Lombardo *et al*., 1999).

VP2- It has long been identified as the host protective antigen as it contains the antigenic region responsible for the induction of neutralizing antibodies and for serotype specificity (Fahey *et al*., 1989) and is the basis for IBDV protection (Azad *et al*., 1987). The genetic basis for the antigenicity of VP2 was determined (Schnitzler *et al*., 1993), and the identification of amino acids responsible for antigenicity was reported by Letzel *et al*., 2007.

VP3- It is a group specific antigen that is recognized by non-netralizing antibodies and it is recognized by monoclonal antibodies directed against VP3 from strains of both serotypes 1 and 2 (Becht *et al.*, 1988). VP4 is involved in the processing of 110 kDa precursor polyprotein (Jagadish *et al.*, 1988).

2.4 Economic importance of IBD

De Wit (2001) indicated direct losses such as reduction of average body weight and increased food consumption (conversion), indirect losses as a consequence of immune suppression caused by Gumboro disease and additional losses by using antibiotics in the treatment of secondary infections. They also emphasized the existence of antibiotic residues in chicken meat, which made it useless for human consumption.

Lukert and Saif (2004) reported that the economic importance of IBD was manifested in two ways: First, some virus strains like vvIBDV caused high mortality in chickens at 3 weeks of age and older. Second, and more importantly, IBDV causes prolonged immunosuppression in chickens infected at an early age, which lead to higher susceptibility to secondary infections and impediment of vaccine responses to vaccines against IBD and other diseases.

2.5 Clinical signs

van den Berg (2000) reported that the IBD is an acute, highly contagious disease of young chickens. Young chicks at the age of 3 to 6 weeks are susceptible to clinical manifestations of the disease.

Office International Des Epizooties (2004) reported that depending on the virulence of the infecting strain and the status of the IBDV specific antibody, the flock may show very high morbidity with severe depression lasting about 5-7 days.

Further mortality starts at the second and third day after infection, reaches a peak at day 4 and then drops quickly.

van den Berg *et al*. (2000) reported that the surviving chickens recover rapidly with a state of apparent health after 5 days.

The mortality rates are highly variable and depend on virulence of the strain, residual MDA levels, as well as the age and breed of the birds (Office International Des Epizooties, 2004).

The main clinical signs are watery diarrhoea, ruffled feathers, reluctance to move, anorexia and prostration (Office International Des Epizooties, 2004). Post-mortem lesions include dehydration of the muscles with haemorrhages, enlargement and discoloration of kidneys with urate crystals in the tubules (Office International Des Epizooties, 2004). The bursa of Fabricius shows the main lesions to be considered for IBD diagnosis.

2.6 Pathology of IBD

Lukert and Saif (2004) reported that the primary target organ of IBDV is the bursa of Fabricius but also other lymphoid tissues may be infected.

In birds that die at the peak of disease outbreak, the bursa is enlarged and swollen with a pale yellow discoloration (Office International Des Epizooties, 2004). Intrafollicular haemorrhages may be present and, in some cases, the bursa may be completely haemorrhagic. Peribursal oedema will be present during early infection (Office International Des Epizooties, 2004).

Lukert and Saif (2004) have reviewed macroscopic and microscopic changes in the bursa of Fabricius and other lymphoid organs. On day 3 post infection, the bursa begins to increase in size and weight because of oedema and hyperaemia. After day 4 the BF begins to recede. By day 5, the BF returns to normal weight but it continues to atrophy. By day 2 to 3 post infection, the BF has a gelatinous yellowish transudate covering the serosal surface. Longitudinal striations on the surface become prominent and the normal colour turns to cream colour. The

transudate disappears as the BF returns to its normal size and the organ becomes gray during the following period of atrophy. The infected BF often shows necrotic foci and at times petechial or ecchymotic haemorrhages on the mucosal surface.

The spleen may be slightly enlarged and very often has small gray foci uniformly dispersed on the surface (Lukert and Saif, 2004). Although lesions in the BF are comparable between vvIBDV or IBDV infected chickens, vvIBDV strains cause a greater decrease in thymic weight index and more severe lesions in the cecal tonsils, thymus, spleen and bone morrow compared to less virulent strains (Lukert and Saif, 2004).

Microsocpical changes are most severe in the BF. As early as 1 day post infection there is degeneration and necrosis of lymphocytes in medullary area of bursal follicles. Lymphocytes are soon replaced by heterophils, pyknotic debris and hyperplastic reticuloendothelial cells (Lukert and Saif, 2004). All lymphoid follicles are affected by 3 or 4 days post infection and the increase in weight of BF seen at this time is caused by severe oedema, hyperaemia and marked accumulation of heterophils (Lukert and Saif, 2004). As the inflammatory reaction declines, cystic cavities develop in the medullary areas of follicles; necrosis and phagocytosis of heterophils and plasma cells occur and fibroplasia is observed in intrafollicular connective tissue (Lukert and Saif, 2004).

2.7 Hematological studies

Limited hematological studies have been carried out in this respect of chickens suffering from IBD.

Chineme and Cho (1984) reported increased mean hematocrit values, as well as lymphocytopenia, in IBDV-infected chickens.

In addition, it has been suggested that hemocoagulopathy is involved in the pathogenesis of IBD (Chineme and Cho, 1984. Kosters *et al.,* 1972. Skeeles *et al.,* 1980 ; Skeeles *et al.,* 1979).

Weiss and Kaufer - Weiss (1994) described the two distinct phases of viremia during IBDV infections and the speculated hemocoagulopathy in the pathogenesis of IBD in chickens as well as the differences in susceptibility of chickens, turkeys and ducks to the disease provide a basis for a comparative hematological study in chickens, turkeys and ducks.

Oladele *et al.* (2005) reported increased PCV, eosinophilia and biphasic lymphopenia and heterophilia developed in chicks after IBD infection.

Dohms *et al.* (1988) revealed that the broilers infected with IBD at 3 weeks of age have a 51 % reduction in plasma cell content at 5-14 days post infection.

2.8 Diagnosis of Infectious Bursal Disease Virus

Conventional diagnosis of IBD relies upon clinical findings, pathological changes, and virological and serological methods such as agar gel precipitation test (AGPT), fluorescent antibody test (FAT), electron microscopy (EM), counter immuno electrophoresis (CIE), serum neutralization test (SNT), indirect enzyme linked immune sorbent assay (ELISA) etc. The conventional methods are time consuming, laborious and less sensitive. Most of very virulent field isolates do not replicate in common tissue culture which is the major problem in virus. The virus-neutralization assay is the only serological test, which can reliably differentiate IBDV antigenic serotypes and subtypes (Jackwood and Saif, 1987), where as for virus neutralization test, the field strains need to be adapted to grow *in vitro*. Furthermore, there is always a risk of modification of antigenic and pathologic

characteristic of the virus during adaptation procedure (Lukert and Saif, 1997). The application of molecular techniques like reverse transcription- polymerase chain reaction (RT- PCR) as a tool for the diagnosis of IBDV infection has been reported (Lee *et al.*, 1994, Jackwood and Jackwood, 1997 and Banda and Villegas, 2004).

2.8.1 Comparison of conventional diagnostic techniques of IBDV

Dash *et al.* (1991) compared the sensitivity and specificity of different tests like AGDT, CIE, indirect fluorescent antibody technique, SNT and ELISA for early detection of the antigen and antibody against IBDV. The antigen was detected from one day to four days PI by AGID, where as CIE detected from 12 hours to six days PI.

Mangala Gowri *et al.* (1997) compared gel precipitation test, CIE, dot ELISA and avidin-biotin dot ELISA for detection of IBDV from bursal samples. They found that there was no difference in the specificity of these tests. AGID and CIE detected 84 and 79 per cent positive cases respectively.

Barman *et al.* (2003) conducted a comparative study for detection of IBDV antigen from BF of suspected birds by AGPT and double antibody sandwich ELISA, which revealed 35.4 and 69.2 per cent positive samples respectively.

Majee *et al.* (2003) screened tissue suspensions for detection of IBDV antigen by AGPT and dot-ELISA. Out of 228 tissue suspensions screened by dot-ELISA, 32 were found positive, where as AGPT detected IBDV in 30 samples.

Parthiban *et al.* (2003) compared AGPT, dot ELISA, SDS-PAGE and electron microscopy for the detection of cell culture adapted IBDV. They concluded that AGPT was unsuitable for detecting cell culture adapted IBDV.

2.8.1.1 Serological tests

An AGID, virus neutralisation (VN) or ELISA may be carried out on serum samples (Office International Des Epizooties, 2004). The infection usually spreads rapidly within a flock of birds. Therefore, only small percentage of the flock needs to be tested to detect the presence of IBDV-specific antibodies.

Lukert and Saif (2004) revealed that the ELISA procedure has the advantage of being a rapid test with the results easily entered into computer software programs. With these programs it is possible to establish an antibody profile on breeder flocks that will indicate the flock immunity level and provide information for developing proper immunization programs for both breeder flocks and their progeny.

Lukert and Saif (2004) reported that the VN test is the only serological test that will discriminate the different serotypes of IBDV and it is still the method of choice to discern antigenic variations between isolates of the virus.

2.8.2 Reverse transcription – polymerase chain reaction (RT-PCR)

2.8.2.1 IBDV RNA extraction

Kataria *et al.* (1999) compared four methods of IBDV RNA extraction (lysis buffer, phenol-chloroform, lithium chloride precipitation by 4M lithium chloride and 8M lithium chloride). They found that RNA extraction by lithium chloride method yielded sufficiently pure RNA for characterization of IBDV.

Sellers *et al.* (1999) extracted IBDV RNA from field isolates of IBDV using guanidinium isothiocyanate method.

Liu (2000) extracted IBDV RNA by two consecutive phenol: chloroform: isoamyl alcohol (25:24:1) extractions.

Parthiban *et al.* (2000) extracted viral RNA from bursal suspensions of the IBDV isolates using Guinidinium acid phenol method.

Banda *et al*. (2001) reported that Single step guanidinium isothiocyanate method was used for extraction of IBDV nucleic acid from frozen bursae.

Kataria *et al.* (2001) extracted total RNA after homogenizing IBDV infected bursal tissue in TNE buffer followed by phenol-chloroform treatment.

Toroghi *et al.* (2001) extracted total RNA from IBDV infected cell cultures by Trizol reagent (Gibco BRL).

Elankumaran *et al.* (2002) detected viral RNA in different tissues of commercial broilers inoculated at one day of age with E/Del variant strain of IBDV. They extracted viral RNA by Trizol Reagent (GIBCO, Life Technologies, USA). The IBDV RNA was detected up to 6 weeks PI from BF, caecal tonsils and bone marrow.

2.8.2.2 Denaturation of double stranded RNA

Complete denaturation of double stranded RNA to convert it into single stranded prior to cDNA synthesis is critical during RT-PCR. Many workers have used denaturants like dimethyl sulphoxide (DMSO) or methyl mercuric hydroxide (MMH), instead of heat denaturation alone in order to increase the sensitivity of the test. Dimethyl sulphoxide (DMSO) was routinely used in the denaturation of IBDV double stranded (ds) RNA for reverse transcription (Azad *et al*., 1985 and Kibenge *et al*., 1990).

Vakharia *et al.* (1992) used methyl mercuric hydroxide (MMH) to denature double stranded RNA prior to cDNA synthesis.

Qian and Kibenge (1994) found heat denaturation at 65ºC for 90 min along with DMSO to be superior to heat denaturation alone at 65ºC for 10 min.

Jackwood *et al*. (1996) reported that the integrity of viral RNA was affected by freezing and thawing and contamination of the sample by other microorganisms.

Mittal *et al*. (2005) reported that Heat denaturation of the double standard RNA (dsRNA) in boiling water bath at 95ºC for 5 min followed by snap chilling in ice prior to reverse transcription without using highly toxic denaturants like methyl mercuric hydroxide (MMH) or dimethyl sulphoxide (DMSO) was found to be sufficient for VP2 gene amplification.

More or less similar observation was also made by Kataria *et al*. (1998). Also the heat denaturation of template cDNA in the reaction mixture prior to addition of *Taq* DNA polymerase improved efficiency and stability of the enzyme over longer cycling period.

Total RNA extracted from infected bursal tissue homogenates by Trizol reagent yielded sufficiently pure RNA for RT-PCR. Random hexanucleotide primers instead of specific primers used for reverse transcription of viral RNA had an advantage of producing random cDNA fragments which could be amplified by any set of the primers. Use of random primers increases the sensitivity of PCR.

2.8.2.3 Polymerase chain reaction

Detection of IBDV in clinical samples is conveniently carried out serologically by AGPT and ELISA using hyperimmune antiserum (Nachimuthu *et al.*, 1995), which is being less sensitive and laborious, are often difficult to interpret. The nucleic acid based detection tests like RT-PCR and nucleic acid hybridization

overcome these problems and therefore have been used for the detection and differentiation of various IBD viruses (Lin *et al.*, 1994 and Kataria *et al.*, 2000). Although the exact molecular basis for the different strains of IBDV is not known, there are genetic markers that distinguish classical and very virulent strain of IBDV. The most studied markers are within a variable region of the gene encoding viral protein VP2. This region is flanked by highly conserved sequence among all type 1 strains of IBDV (Cavanagh, 2001).

Lin *et al.* (1993) amplified variable cDNA regions in VP2 gene of five highly virulent IBDV isolates of Japan by PCR. Two primers were used for cDNA synthesis and PCR to generate 474 bp amplicon.

Jackwood and Jackwood (1994) performed RT-PCR using oligo primers specific to VP2 of IBDV on cell culture harvested seven IBDV isolates, which generated 394 bp product.

Lee *et al.* (1994) used single-tube, noninterrupted RT-PCR for the detection of IBDV using a primer set framing a region within the gene coding for IBDV VP2 protein to amplify a 318 bp fragment of the IBDV genome.

Liu *et al.* (1994) performed RT-PCR using primer set P1 (5' - TCA CCG TCC TCA GCT TAC - 3') and P2 (5' - TCA GGA TTT GGG ATC AGC - 3') specific to VP2 region of IBDV and generated the fragment of 643 bp for all the isolates (Dormitorio *et al.*, 1997; Parthiban and Thiagarajan, 2000; Kataria *et al.*, 2001 and Kataria *et al.*, 2004).

Stram *et al.* (1994) performed RT-PCR on tissue culture adapted virus and BF of infected chickens to identify Israeli isolates of IBDV. Two pairs of primers

were used to amplify virus specific sequences from the VP2 and VP3 genes yielding products of 365 bp and 320 bp respectively.

Jackwood *et al.* (1996) obtained 394 bp fragment amplicon in RT-PCR assay on experimentally infected and phenol: chloroform treated bursal tissues for detecting IBDV RNA.

Tham *et al*. (1995) developed RT-PCR assay in which two pairs of primers were designed to amplify the 5'- and 3'-termini of segment A genes. The identity of both amplified fragments 309-bp fragment and 520-bp fragment were confirmed by restriction endonuclease analysis, chemiluminescence, southern blot hybridization and direct cycle sequencing.

Jackwood and Jackwood (1997) examined 22 IBDV strains using RT-PCR, thereby amplifying a 394 bp fragment of the VP2 gene, which was tested for six different restriction sites.

Jackwood and Sommer (1997) performed RT-PCR on 26 strains of IBDV by targeting VP2 coding region which had generated 743 bp amplicon.

Chen *et al.* (1998) amplified VP2 region of IBDV genome from nine Mainland Chinese strains by RT/nested PCR using two sets of primers. The outer primers (P1 and P2) generated an amplicon of 643 bp and the inner primers (P3: 5' – GCC CAG AGT CTA CAC CAT AAC TGA – 3'; P4: 5' – GCG ACC GTA ACG ACA GAT CC – 3') annealed to the complement of bases 611- 630 and bases 1081–1100 of ORFA–1, respectively.

Liu *et al.* (1998) developed a rapid and sensitive RT-PCR protocol for the detection and amplification of IBDV RNA from infected BF. Four primers were

selected from the sequence of a hypervariable region in VP2 genes. For the amplification of genomic IBDV RNA, the product (643 bp) of RT-PCR was reamplified and double checked by a nested PCR amplifying 491 bp cDNA. The sensitivity of nested PCR was at least 100 times greater than RT-PCR.

Pitcovski *et al.* (1998) determined the sequence of the coding region of segment A, coding for the viral proteins (VPs) VP2, VP4, and VP3 of a very virulent IBDV isolated in Israel. They used separate primers for performing RT-PCR for each viral protein.

Sellers *et al.* (1999) amplified 847 bp amplicons in VP2 gene from three field isolates of IBDV using primers (REIBD5' : 5' – CAA CAG CCA ACA TCA ACG AC – 3' and REIBD3' : 5' – CAC CTC CAT GAA GTA CTC AC – 3').

Parthiban *et al.* (2000) generated 474 bp corresponding to a VP2 region of IBDV genome using RT PCR.

Yamaguchi *et al.* (2000) detected change in the sequence of VP2 hypervariable region by RT-PCR during serial passage of IBDV vaccines in chickens. Such changes could be ascertained by RT-PCR which was found useful for diagnosis and differentiation of IBDV.

Banda *et al.* (2001) performed RT-PCR to amplify a 248 bp product corresponding to the hypervariable region of IBDV VP2 gene. They also performed RFLP for differentiating the strains of IBDV particularly vvIBDV.

Chai *et al.* (2001) performed one step RT-PCR on cell culture adapted IBDV using the oligonucleotide primers designed to amplify a 555 bp fragment of the VP2

gene. The primers were PF 5’ - TGA GAC TTG GTG ACC CCA TAC - 3’; and PR 5’ - CGA CGG ATC CTG TTG CCA CTC - 3’.

Kataria *et al.* (2001) described single-tube uninterrupted RT-PCR for direct detection of IBDV in clinical samples by amplifying the hypervariable region of the VP2 gene sequence. The VP2 gene of IBDV was amplified to generate the products of 643 and 500 bp for all the five isolates of IBDV.

Jackwood and Sommer (2002) performed RT-PCR on viral RNA to amplify VP2 gene of 743 bp and further used it as a genetic molecular marker for differentiation of wild-type IBDV and virulent vaccine strains.

Viswas *et al.* (2002) amplified hypervariable region of IBDV VP2 gene by RT-PCR to obtain 474 bp product.

Bais *et al.* (2003) reported a strategy to amplify the complete coding sequence of IBDV genome segments A and B of an Indian very virulent field isolate, in overlapping fragments by RT-PCR and cloned in pGEMT vector system for molecular characterization.

Kataria *et al.* (2004) performed RT-PCR on seventeen bursal samples suspected for IBD which were collected from different parts of India. Amplicon of 643 bp was generated in 14 samples.

CHAPTER-III

Materials and Methods

CHAPTER – III

MATERIALS AND METHODS

The present study was conducted in the Department of Veterinary Pathology, College of Veterinary Science and Animal Husbandry, Anjora, Durg, Chhattisgarh to study prevalence, pathology and molecular diagnosis of infectious bursal disease in broilers. The data of prevalence of IBD were collected from Durg, Rajnandgaon and Raipur district of Chhattisgarh.

3.1 Collection of data

The studies on prevalence and pathology of Infectious Bursal Disease in broiler birds of Chhattisgarh was undertaken in various commercial broiler farms of Durg, Rajnandgaon and Raipur districts of Chhattisgarh state.

During the visit of different poultry farms of Durg, Rajnandgaon and Raipur districts following data with respect to IBD were collected.

- Total strength of birds in poultry farm.
- Number of affected birds showing clinical signs of IBD.
- Number of birds reported dead showing postmortem lesions suggestive of IBD.
- Vaccination status etc.

3.2 Clinical samples

Tissue samples from bursa of Fabricius, liver, spleen, kidney, heart, thymus and brain showing lesions suggestive of IBD were collected for pathological investigation.

3.3 Organ weight factor

Liver and spleen of suspected IBD affected birds were collected and weighed using an electronic balance. The body weight of these birds were also taken for calculation of organ weight factor as per the following formula

$$\text{Organ weight factor} = \frac{\text{Organ weight}}{\text{Whole body weight}} \times 1000$$

3.4 Pathology

3.4.1 Gross pathology

All the carcasses collected from different poultry farms were routinely subjected to post - mortem examination, paying attention to age of flock, the lesions of bursa of Fabricius, kidneys, spleen, thymus etc. and haemorrahages in thigh, breast muscle and junction between proventriculus and gizzard.

3.4.2 Histopathology

Representative tissue pieces from the affected liver, kidneys, spleen, brain, bursa of Fabricius and thymus were collected in 10% formal saline for histopathological examination. The routine procedure adopted at Department of Veterinary Pathology, College of Veterinary Science and Animal Husbandry, Anjora, Durg was used for section cutting Tissue sections were cut between 3-5µ and stained with Haematoxylin and Eosin stain (H & E) as described by Culling (1963).

3.5 Haematological study

Ten Blood samples from jugular vein were collected in sterilized evacuated heparinised (Heparin @ 20 I.U. / ml of blood) tubes from IBD infected birds of each flock showing signs suggestive of IBD from Durg, Rajnandgaon and Raipur district.

Ten blood samples from normal healthy birds were also collected to compare the data of IBD affected birds.

The heparin admixed blood samples were used for haemoglobin (Hb), packed cell volume (PCV), total erythrocyte count (TEC) and total leucocyte count (TLC) using conventional methods. Haemoglobin (Hb) content of blood was determined by Cyanmet haemoglobin method and expressed in g/dl. Packed cell volume was estimated according to Jain (1986) and expressed in percent. Total erythrocyte count and Total leucocyte count were done as per the method described by Nambiar (1960) using diluting fluid recommended by Natt and Herrick (1954) and the values were expressed in million/µl and thousands/µl respectively. For differential counts, thin blood smears were air dried, fixed in methanol for three minutes and stained with Giemsa stain. The percentage of different leucocytes were determined by examining the stained smear under oil immersion of light microscope and a total of 100 white blood cells were identified and counted.

3.6 Enzymed linked immunosorbent assay test

3.6.1 Collection of serum sample

Jugular blood samples were collected in dry evacuated tubes from IBD affected flocks and tube of blood was allowed to clot and the separated serum was utilized for ELISA test.

3.6.2 Description of test:

The IBD ELISA kit was used to measure the amount of antibody of IBDV in the serum of chickens. Microtitre plates were pre-coated with inactivated IBDV antigen. Chicken serum samples ware diluted and added to the microtitre wells where anti-IBD antibodies present was bound and form an antigen - antibody complex. Non specific antibodies and other serum proteins were then washed away. Antichicken IgG labelled with the enzyme alkaline phosphatase is then added to the wells and binds to chicken anti-IBD antibodies bound to the antigen. After another wash unreacted conjugate were removed, substrate was added in the form of pNPP chromogen. A yellow colour developed in the anti-IBD antibody containing samples and the intensity was directly related to the amount of anti-IBD present in the sample.

Reagents used:

i. **IBD Coated plates**. Inactivated viral antigen on microtitre plates.

ii. **Conjugate reagent** Anti-Chicken: Alkaline Phosphatase in Tris buffer with protein stabilisers, inert red dye and sodium azide preservative (0.1% w/v).

iii. **Substrate tablets**. PNPP (p-Nitrophenyl Phosphate) tablets to dissolve with Substrate buffer.

iv. **Substrate buffer reagent**. Diethanolamine buffer with enzyme co-factors.

v. **Stop solution**. Sodium Hydroxide in Diethanolamine buffer.

vi. **Sample diluent reagent**. Phosphate buffer with protein stabilisers and sodium azide preservative (0.1% w/v).

vii. **Wash buffer**. Powdered Phosphate Buffered Saline with Tween.

viii. **Negative control**. Specific Pathogen Free serum in Phosphate buffer with protein stabilisers and sodium azide preservative (0.1% w/v).

ix. **Positive control**. Antibodies specific to IBD in Phosphate buffer with protein stabilisers and sodium azide preservative (0.1% w/v).

Materials and Equipment required

i. Precision Pipettes and disposable tips.

ii. 8 or 12 channel pipette / repeater pipette.

iii. Plastic tubes for sample dilution.

iv. Distilled or deionised water.

v. Microtitre Plate Reader with 405 nm filter.

vi. Microtitre Plate Washer.

Reagent preparation

i. **Substrate reagent** To make substrate reagent, 1 tablet to 5.5 - 6 ml of substrate buffer were added and allowed to mix until fully dissolved. The prepared reagent was made on day of use. Tablets were droped into clean container and added appropriate volume of Substrate Buffer.

ii. **Wash buffer**. Emptied the contents of one wash buffer sachet into one litre of distilled water and were allowed to dissolve fully by mixing.

iii. All other kit components are ready to use but were allowed to come to room temperature 27 °C before use.

Sample preparation

1. Each test sample was diluted 1:1500

Positive And Negative Kit Controls Do Not Require Diluting

Test procedure

i. IBD coated plate was removed from sealed bag and recorded location of samples on template.

ii. 100 µl of negative control was added into wells A1 and B1.

iii. 100 µl of positive control was added into wells C1 and D1.

iv. 100 µl of diluted samples was added into the appropriate wells. the plate was Covered with lid and incubated at room temperature 27°C for 30 minutes.

v. Contents of wells were aspirated and washed 4 times with wash buffer (350µl per well). The plate was inverted and taped firmly on absorbent paper.

vi. 100 µl of Conjugate reagent were added into the appropriate wells. The plate was covered with lid and incubated at room temperature 27°C for 30 minutes.

vii. Contents of wells were aspirated and washed 4 times with wash buffer (350µl per well). The plate was inverted and taped firmly on absorbent paper.

viii. 100 µl of substrate reagent was added into the appropriate wells. Plate was covered with lid and incubated at room temperature 27°C for 15 minutes.

ix. 100 µl of stop solution were added to appropriate wells to stop reaction.

x. Blank the microtitre plate reader on air and recorded the absorbance of controls and samples by reading at 405 nm.

3.7 Molecular diagnosis of infectious bursal disease virus by Reverse Transcription - Polymerase Chain Reaction (RT-PCR)

3.7.1 Collection of clinical samples from IBD affected birds for RT-PCR

Bursa and Spleen from the affected birds showing lesions suggestive of IBD was collected from different poultry farms. These samples were processed for confirmation of IBD Virus by RT-PCR. RT-PCR was performed using two different sets of primers (Table.1). Both the primers amplify the middle third of the VP2 gene in segment A of Serotype 1 IBDV.

3.7.2 Method of RT - PCR

Total RNAs were extracted and purified using Viral RNA Extraction Kit (Ambion). RT-PCRs were performed with a One-Step RT-PCR Kit (Qiagen) according to manufacturer's instruction. Template RNA, primer solutions, dNTP Mix, 5x QIAGEN OneStep RT-PCR Buffer, and RNase-free water were thawed and placed on to the ice. Master Mix was prepared as given in (Table.2). The master mix was mixed thoroughly and was dispensed in appropriate volumes into PCR tubes. Template RNA (2 µg/reaction) was added to the individual PCR tubes and Used in a Programmed thermal cycler (Table. 3).The PCR tubes were placed in the thermal cycler. A 5 µl RNA (approximately containing 5µg of RNA) was denatured at 95^0C for 5 minutes and then used for the synthesis of cDNA using one step RT-PCR kit (Qiagen) according to the manufacturer's instructions. Briefly, to 5 µl of RNA, 1ml of primer (0.2 µg/ µl) and 12 µl deionized DEPC treated water (nuclease free) were added and incubated at 70^0C in water bath for five minutes. Then 5X reaction buffer, 20 U of Ribonuclease inhibitor and 2 µl of dNTPs (10mM) were added and incubated at 25^0C for five minutes. Finally, 200U

Table 1. Details of primers used in RT-PCR

PRIMER	PRIMER SEQUENCE	LOCATION OF PRIMER	EXPECTED AMPLICON SIZE	REFERENCES
Forward	5'TCACCG TCCTCAG CTTAC3'	587 - 604	643	(Liu *et al.*,1994)
Reverse	5'TCAGGA TTTGGGA TCAGC3'	1212 - 1229		
Forward	5'CCCAGA GTCTACA CCATA3'	703 - 720	474	(Lin *et al.*, 1993)
Reverse	5'TCCTGT TGCCACT CTTTC3'	1159 – 1176		

Table 2. Composition of master mix.

Component of master mix	**Volume/reaction**	**Final concentration**
RNase-free water (provided)	Variable	–
5x QIAGEN OneStep RT-PCR Buffer*	10.0 µl	1x
dNTP Mix (containing 10 mM of each dNTP)	2.0 µl	400 µM of each dNTP
Primer A	Variable	0.6 µM†
Primer B	Variable	0.6 µM†
QIAGEN OneStep RT-PCR Enzyme Mix	2.0 µl	–
RNase inhibitor (optional)‡	Variable	5–10 units/reaction
Template RNA, added at step 4	Variable	1 pg – 2 µg/reaction
Total volume	**50.0 µl**	**–**

Table 3. Steps and conditions of thermal cycling for RT- PCR

Reverse transcription	30 min	50°C
Initial PCR activation step	15 min	95°C
3-step cycling		
Denaturation	30 sec	95°C
Annealing	30 sec	54.3°C
Extension	1 min	72°C
Number of cycles	40	
Final extension	10 min	72°C

of M-MuLV RTwas added and incubated at 25^{0}C for ten minutes and then at 42^{0}C for one hr. The reaction was stopped by heating at 70^{0}C for ten minutes, chilled on ice and stored at -20^{0}C until used for PCR. Amplified bands were visualized under UV light at a wavelength of 254 nm with Eagle Eye Gel Documentation System after ethidium bromide staining @ 0.5µg/ ml. The size of the bands was confirmed with the help of a 100-bp DNA ladder as a molecular size marker.

3.8 Statistical analysis

Statistical analysis of data was done using Indipendent‘t’ test (Snedecor and cochran., 1968).

CHAPTER-IV

Results and Discussion

CHAPTER – IV

RESULTS AND DISCUSSION

Poultry sector has strong presence in Chhattisgarh state. Commercial and backyard poultry are in two major sectors. The commercial poultry sector has layer and broiler which are growing enterprise. Broiler birds showing clinical signs and postmortem lesions suggestive of IBD of commercial poultry farms of Durg, Rajnandgaon and Raipur districts were used for the present study during the period February 2012 to October 2012. The broiler birds brought for post- mortem examination at Department of Veterinary Pathology, College of Veterinary science and Animal Husbandary, Anjora, Durg showing lesions suggestive of IBD were also used for the present study.

4.1 Prevalence of infectious bursal disease

The present study consisted of prevalence of IBD in the selective poultry flocks in Durg, Rajnandgaon and Raipur districts of Chhattisgarh (Table.4). The poultry flocks in Chhattisgarh were vaccinated with live intermediate vaccine or also called as Georgia strain. Commercial poultry farms of Durg, Rajnandgaon and Raipur districts are densely populated and total capacity of poultry farms are quite large where majority of broiler birds in the farms are housed in the deep litter system. The data regarding prevalence of IBD were collected during the period February 2012 to October 2012. Visits were made to the farms and the birds died during the period were collected and subjected to post mortem examination paying attention to the lesions suggestive of IBD. In the present study maximum mortality due to IBD was reported in Durg district and minimum mortality was reported in Raipur district.

Present study revealed mortality which ranged from 8 to 12% in the age group of 3 to 6 weeks of broilers. The present findings were closely corroborated with the findings of the Sah *et al*. (1995). In contrast to present findings, Jaisankar *et al.* (2003) reported that the disease was more prevalent in birds of 0 -5 weeks of age (94.06%). As per the information recorded from the farmer, the mortality rates due to IBD were 12%, 10% and 8% respectively in Durg, Rajnandgaon and Raipur but OIE Terrestrial Manual (2008) reported mortality of 5 to 10% which may reach 30 to 40% in broiler due to IBD. Saif *et* al. (2000) reported 1.5 and 30% mortality due to IBD in native and broiler flocks, respectively. In the present study highest and lowest mortality were reported as 12% and 8% at Durg and Raipur district, respectively.

In the present study it has been observed that IBD not only produced huge mortality, but it also caused immunosuppression and made the birds susceptible to other diseases leading to higher economic losses in commercial farms.

Table 4. Prevalence of IBD in Durg, Rajnandgaon and Raipur districts of Chhattisgarh

DISTRICT	POULTRY FARMS	FLOCK SIZE	MORTALITY NO. (% MORTALITY)	AGE	VACCINATION STATUS
DURG	VCF	12000	1600 (13.3%)	26 DAYS	GEORGIA STRAIN
	VCR	12000	1380 (11.5%)	29 DAYS	GEORGIA STRAIN
	VCK	12000	1420 (11.8%)	34 DAYS	GEORGIA STRAIN
	HDF	1200	117 (9.75%)	29 DAYS	GEORGIA STRAIN
	LITIA	1000	111 (11.1%)	36 DAYS	GEORGIA STRAIN
	BM	1000	113 (11.3%)	30 DAYS	GEORGIA STRAIN
	BF	1000	97 (9.7%)	31 DAYS	GEORGIA STRAIN
	GPF	2000	226 (11.3%)	38 DAYS	GEORGIA STRAIN
RAJNANDGAON	VIF	1200	130 (10.8%)	25 DAYS	GEORGIA STRAIN
	ADF	1000	98 (9.8%)	19 DAYS	GEORGIA STRAIN
	LPF	1000	92 (9.2%)	24 DAYS	GEORGIA STRAIN
RAIPUR	LB	2300	173 (7.5%)	24 DAYS	GEORGIA STRAIN
	LM	2300	180 (7.8%)	25 DAYS	GEORGIA STRAIN
	SF	1200	129 (10.7%)	24 DAYS	GEORGIA STRAIN
	AF	1000	69 (6.9%)	25 DAYS	GEORGIA STRAIN
	VIF	1000	73 (7.3%)	24 DAYS	GEORGIA STRAIN

Note: Name of the farms is not being disclosed because of the fact that commercial farms owners were reluctant to disclose the status of IBD in their farms.

4.2 Organ Weight Factor

A total of 53 bursa of Fabricius and 53 spleen samples of IBD infected birds were collected for the Organ weight study. Simultaneously each of bursa of Fabricius and spleen of normal healthy broiler birds were also collected for statistical analysis of the data. The effect of infectious bursal disease on bursa of Fabricius and spleen in commercial broiler birds are presented in Table 5 and Fig. 1. The mean organ weight factor of healthy and IBD infected bursa of Fabricius were 0.66 ± 0.07 and 0.44 ± 0.04 respectively. The reduction in weight of bursa of Fabricius was due to its atrophy. The mean organ weight factors of spleen were 1.86 ± 0.17 and 0.97 ± 0.10 for healthy and diseased birds respectively. There was significant reduction in the weight of the spleen of IBD infected birds compared to the spleen of healthy birds.

Table 5. Effect of infectious bursal disease on organ weight of bursa of Fabricius and spleen (mean ± SE)

TYPE OF BIRDS	ORGAN WEIGHT FACTOR	
	BURSA	SPLEEN
NORMAL	0.66 ± 0.07	1.86 ± 0.17
DISEASED	0.44 ± 0.04**	0.97 ± 0.10**

N.B. - Superscripts may read column wise for comparison of means. ****** represents $P < 0.01$

4.3 Clinical sign

Commercial broiler farms in Durg, Rajnandgaon and Raipur district of Chhattisgarh formed the basis of the present study. As per the history recorded from the farmer, the affected broilers exhibited anorexia, marked depression, yellowish white diarrhea and soiled vent feathers with prostration. Some affected birds showed the signs of vent pecking, trembling, lameness and staggering gait. These findings were in accordance with the findings of Chowdhury *et al*. (1996) who reported clinical signs of anorexia, depression, ruffled feathers, diarrhoea leading to death in the IBD affected birds. Similar observations were also found by the previous workers (Cosgrove, 1962 and Landgraf *et al*., 1967).

4.4 Gross pathology

At necropsy, the carcasses were found either in good flesh or highly dehydrated and emaciated. Congestion of the subcutaneous blood vessels was frequently seen. Petechial and ecchymotic haemorrhages and congestion were often observed in the leg (Fig. 2), thigh muscle (Fig. 3) and breast muscles. In most cases the bursa of Fabricius was swollen (Fig. 4) and oedematous with creamy or yellowish discoloration. Gelatinous exudates were frequently found in the serosa of the bursa. Congestion and haemorrhages on the serosal and mucosal surfaces of the bursa (Fig. 5) were also noticed in few cases. Occasionally, the bursa became atrophied and whitish or creamy, which sometimes contained cheesy mass in the lumen (Fig. 6). The spleen of some IBD infected birds were swollen and mottled (Fig. 7) but atrophied and pale in others (Fig. 8). The thymus was found necrosed in most of the cases. Few chicks had swollen and pale kidneys (Fig. 9). The liver of affected broilers had congestion and pale areas in some cases. Haemorrhages in the caecal tonsils and at the junction between proventriculus and gizzard (Fig. 10) were seen in few cases.

Present findings of swelling and paleness of kidneys were also recorded by Baxendale (2002). Eterradossi and Saif (2008) also reported swelling and pallor with heavy accumulation of urates in the tubules and ureters of kidneys. Hanson (1962) recorded hemorrhages in thigh and pectoral muscles. Necropsy findings in spleen, thymus and bursa of Fabricius were in accordance with the findings of Lukert and Saif (2003). Present findings of haemorrhages in the caecal tonsils and junction between proventriculus and gizzard were in close conformity with the findings of Eterradossi and Saif (2008). Office International Des Epizooties (2004) opined that at the peak of disease outbreak, the bursa was enlarged and swollen with a pale yellow discoloration, intrafollicular haemorrhages and peribursal oedema.

4.5 Histopathology

4.5.1 Bursa of Fabricius

Severe depletion of lymphocytes and necrosis was the main histopathological lesion in the bursa of Fabricius (Fig. 11 and 12). The bursal follicles contained serous exudate (Fig. 13). In few cases the depleted lymphocytes were replaced by proliferating reticuloendothelial cells. Almost complete depletion of lymphocyte resulting in atrophy of follicles, fibroplasia around the follicles and cystic spaces within the follicles were observed. In the present study severe changes were found in the bursa of Fabricius which coincided the observation of Cheville (1967) who described that degeneration and necrosis of individual lymphocytes in the medullary region of the bursa occur as early as day 1 post infection. Lymphocyte degeneration is accompanied by nuclear pyknosis and formation of lipid droplets in the cytoplasm. Lukert and Saif (2004) also reported degeneration and necrosis of lymphocyte in the medullary area of bursal follicles. Lymphocytes were soon replaced by heterophils, pyknotic debris and hyperplastic reticuloendothelial cells.

4.5.2 Spleen

Lymphoid depletion of various degrees was found in the spleen (Fig. 14) of affected broiler birds. It was occasionally associated with congestion, haemorrhage and thickening of the arterial wall. The present finding of lymphocytolysis in spleen corroborated the findings of Lukert and Saif (2003) who reported that the spleen showed hyperplasia of the reticuloendothelial cells around the adenoide sheath arteries during the early stage of infection. Lymphoide necrosis occurs in the periarteriolar lymphoide sheath by 3 days post infection.

4.5.3 Kidney

In the present study kidneys revealed haemorrhage in the interlobular capillaries (Fig. 15), degenerative and necrotic changes of proximal and distal convoluted tubules (Fig. 16) leading to atrophy of glomerulus (Fig. 17). Similar findings were also reported by Chowdhury *et al*. (1996) who reported nephritic lesions including degeneration, dissociation and coagulation necrosis of tubular epithelial cells. Mondal *et al.* (2008) also reported congestion, degenerative changes and necrosis of convoluted tubules.

4.5.4 Heart

In the present study no significant changes were observed in the heart of IBD infected birds.

4.5.5 Lung

In the present study severe pneumonic changes like thickening of interlobular septa due to heavy infiltration with mononuclear cells (Fig. 18), severe congestion leading to stage of grey hepatization (Fig. 19) and serofibrinous exudation in the bronchiole (Fig. 20) were observed in few cases. The changes observed in the present study might be due to immunosuppression caused by the IBDV leading to secondary bacterial infection as also reported by Aricibasi (2010) who found beside lysis of dividing B cells, virus also effects

macrophage function leading to immunosuppression. IBD can cause substantial direct losses in affected flocks, but it also affects the ability of infected birds to develop immunity to other diseases.

4.5.6 Brain

In the present study brain revealed congestion (Fig. 21) and oedematous changes (Fig. 22) in the IBD infected birds. The literature on the effect of IBDV in brain is scanty.

4.5.7 Liver

In the present study liver revealed haemorrhage (Fig. 23) and severe necrosis of hepatocytes (Fig. 24). Chowdhury *et al*. (1996) observed congestion in the central vein, fatty changes or diffuse coagulative necrosis of hepatocyte. Mondal *et al*. (2008) also reported coagulative necrosis of hepatocytes, along with infiltration of mononuclear cells in the portal areas of the liver of IBD affected birds.

4.5.8 Thymus

In the present study IBD affected broilers had severe medullary lymphocytic depletion (Fig. 25 and 26) along with haemorrhage and congetion (Fig. 27) in the thymus. Almost similar changes were also observed by Inoue *et al.* (1994) who reported the changes in the thymus shortly after infection and include areas of lymphoid necrosis and hyperplasia of the reticular and epithelial components in the medullary region of thymic follicles.

4.6 Haematological studies

The results of haematological changes have been presented in Table 6 and graphically depicted in Fig. 28 to 36.

4.6.1 Hemoglobin values

The result of hematological investigation revealed significant ($P \leq 0.05$) increase in the hemoglobin values (gm/dl) in all the three IBD infected flocks (Durg - 10.59 ± 0.13, Rajnandgaon - 10.37 ± 0.11 and Raipur -10.32 ± 0.16) as compared to the normal healthy broilers (9.8 ± 0.17). Increased hemoglobin values, as observed in infected chickens in this study, have previously been observed by Chineme and Cho (1984). Finding of the present study is also supported by Oladele *et* al. (2005). Hyperhemooglobinemia of the present investigation might be due to dehydration, which was one of the important clinical and postmortem findings in IBD.

4.6.2 PCV values

The present investigation revealed significant ($P \leq 0.01$) increase in the PCV values (%) in all the IBD infected flocks (Durg - 29.43 ± 0.39, Rajnandgaon - 29.26 ± 0.45 and Raipur - 29.02 ± 0.42) in comparision to the normal healthy broilers (25.54 ± 0.95). Similar changes were observed by Oladele *et al.* (2005) who reported PCV values of chicks slightly increased generally from a pre-infection level of 27% to 30.5% at 6 hours post infection and 31% at 12,120 and 144 hours post infection.

4.6.3 TEC and TLC values

The result of hematological investigation revealed non significant increase in the TEC values (10^6/µl) in all the IBD infected flocks (Durg - 3.054 ± 0.05, Rajnandgaon - 3.001 ± 0.11 and Raipur - 3.011 ± 0.05) compared to the normal healthy birds (2.85 ± 0.04).The present study showed significant ($P \leq 0.01$) increase in the TLC values (10^3/µl) in all the three farms (Durg - 28.3 ± 0.92, Rajnandgaon -26.7 ± 0.85 and Raipur - 19.9 ± 0.38) compared to the normal healthy birds (19.9 ± 0.44). Similar changes were observed by Oladele *et al* (2005) who recorded decreased TLC from a preinfection value of 27.7 x

103/µl to 17.33 x 103/µl at 24 hours post infection. It subsequently increased above the preinfection level and peaked at 120 h postinfection with a value of 66.83 x 103/µl, followed by 50.5 x 103/µl at 144 h postinfection.

4.6.4 Differential leucocyte count

The present study showed significant ($P \leq 0.01$) increase in the heterophil percentage in all the three flocks (Durg - 19.5 ± 1.12, Rajnandgaon - 22 ± 0.47 and Raipur - 19.6 ± 1.08) compared to the normal healthy birds (16.1±0.62). Similar changes were observed by Oladele *et al* (2005) who reported that heterophil counts in chicks remained fairly constant up to 48 hours post infection and then rapidly increased to a maximum of 48.08 x 103/µl at 120 h post infection. The result of hematological investigation revealed significant ($P \leq 0.01$) decrease in the lymphocyte percentage in all the three farms (Durg - 64.8 ± 1.87, Rajnandgaon - 63.1 ± 1.42 and Raipur - 62.7 ± 1.40) compared to the normal healthy birds (73.2 ± 0.89). Similar changes were observed by Oladele *et al* (2005) who also reported that lymphopenia was developed in chicks from 24 to 96 hours post infection. According to Campbell and Coles (1986), lymphopenia occurs in acute viral diseases due to glucocorticoid excesses. Specifically, IBDV causes destruction of B-lymphocytes within the bursa of Fabricius, which is the organ of maturation and differentiation of B-lymphocytes, before they migrate into the blood stream. Weiss and Kaufer-Weiss (1994) detected IBDV antigen in the bursa of Fabricius of chickens 11 h postinfection. Thus, 24 hours provide an adequate period for the effect of B-lymphocyte destruction to be evident in the blood in cases where an IBDV infection has been established. Significant ($P \leq 0.01$) decrease in the Eosinophil percentage in all the IBD infected flocks (Durg - 0.8 ± 0.25, Rajnandgaon - 0.9 ± 0.23 and Raipur - 1.2 ± 0.20) was observed in comparison to the normal healthy birds (3.4±0.34). Non significant changes were observed in the basophil percentage in all the three

farms (Durg - 1.7 ± 0.15, Rajnandgaon - 1.7 ± 0.15 and Raipur - 1.9 ± 0.10) as compared to the normal healthy birds (1.5 ± 0.17). Significant (P≤0.01) increase in the monocyte percentage in all the three farms was observed (Durg -13.2 ± 0.59, Rajnandgaon -14.4 ± 0.40 and Raipur -14.5 ± 0.43) as compare to the normal healthy birds (5.8 ± 0.51).

Table 6: Average (Mean ± S.E.) haematological Values of IBD infected broiler flock

PARAMETERS	NORMAL HEALTHY BIRDS	DURG	RAJNANDGAON	RAIPUR
HB (GM/DL)	9.8 ±0.17^{A}	10.59±0.13*^{B}	10.37±0.11*^{B}	10.32±0.16*^{B}
PCV (%)	25.54±0.95^{A}	29.43±0.39**^{B}	29.26±0.45**^{B}	29.02±0.42**^{B}
TEC (10^{6}/µL)	2.85±0.04^{A}	3.05±0.05^{A}	3.00±0.11^{A}	3.01±0.05^{A}
TLC (10^{3}/µL)	19.9±0.41^{A}	28.3±0.92**^{B}	26.7±0.85 **^{B}	19.9±0.38^{A}
EOSINPHIL (%)	3.4±0.34^{A}	0.8±0.25**^{B}	0.9±0.23**^{B}	1.2±0.20**^{B}
MONOCYTES (%)	5.8±0.51^{A}	13.2±0.59**^{B}	14.4±0.40**^{B}	14.5±0.43**^{B}
BASOPHIL (%)	1.5±0.17^{A}	1.7±0.15^{A}	1.7±0.15^{A}	1.9±0.10^{A}
HETEROPHIL (%)	16.1±0.62^{A}	19.5±1.12**^{B}	22±0.47**^{B}	19.6±1.08**^{B}
LYMPHOCYTE (%)	73.2±0.89^{A}	64.8±1.87**^{B}	63.1±1.42**^{B}	62.7±1.40**^{B}

Values indicate Mean ± S.E.

Superscripts may read row wise for comparison of means. NS - No significant difference (*P≤0.05) and (P≤0.01)**

4.7 Enzyme linked immunesorbent assay test

The ELISA test in the present study revealed antibody titer which ranged from 3689 -7974 (mean titer 5602 ± 567.86), 4976 - 7393(mean titer 6243 ± 348.36) and 4990 – 6516 (mean titer 5522 ± 343.00) in affected broilers of Durg, Raipur and Rajnandgaon respectively (Table 7). Homer *et al.* (1992) also observed antibody titers in broilers with a mean range of 3,156 - 4,945 at day 1 of age, with mean titers decreasing below 1,000 at 16 days of age, and then beginning to increase by 28 or 32 days of age until reaching a mean range of 2,418 - 6,173 at 41 days of age. Thus it appeared that the higher antibody titer range observed in the present study was due to IBD field virus (Fig. 37).

Table 7. Mean antibody titer of IBD affected flock

DISTRICT	**DURG**	**RAJNANDGAON**	**RAIPUR**
MEAN TITER	**5602 ± 567.86**	**5522 ±343.00**	**6243 ± 348.36**

For diagnosis purposes testing by the ELISA method is most popular because of easy availability of commercial kits and the test is also faster. In the present study ELISA appears to be a sensitive test for assessing the antibody titer against IBDV.

4.8 Molecular diagnosis of infectious bursal Disease by reverse transcriptase Polymerase Chain Reaction:

The bursae of Fabricius and spleens of broilers of Durg, Rajnandgaon and Raipur showing lesions suggestive of IBD were collected for RT-PCR using two sets of primers (Lin *et al.*, 1993 and Liu *et al.*, 1994).

A total of 53 samples were taken for the detection of virus by using two different sets of primers (Lin *et al.*, 1993 and Liu *et al.*, 1994) to amplify variable portion of VP2 gene to obtain 643 bp products. Out of 53 IBD affected samples of bursa and spleen 45 samples produced an amplicon of 643bp (Fig. 38) using Liu *et al.* (1994) indicating positive result (84.9%) for IBDV (Table. 8). The primer of Lin *et al.* (1993) could not be used due to some mistake in the primer synthesis. In Durg district, out of 35 samples 33 samples (94%) were found positive for IBDV. In Rajnandgaon district, out of 8 samples 4 samples (50%) were found positive for IBDV. In Raipur district, out of 11 samples 9 samples (81%) were found positive for IBDV. Thus it shows Durg district had higher number of IBD positive cases by RT-PCR as compared to Rajnandgaon and Raipur district.

Table 8. Results of RT-PCR using clinical samples of bursa and spleen from the IBD suspected flock

TOTAL NO. OF SAMPLES	POSITIVE CASES	NEGATIVE CASES	TOTAL POSITIVE CASE DETECTED IN RT-PCR
53	**45**	**8**	**84.9%**

Liu *et al.* (1998) developed rapid and sensitive RT-PCR protocol for the detection and amplification of IBDV RNA from infected bursa of Fabricius. Four primers were selected from the sequence of a hypervariable region in VP2 genes. For the amplification of genomic IBDV RNA, the product 643 bp of RT-PCR was reamplified and double checked by a nested PCR amplifying 491 bp cDNA. The sensitivity of nested PCR was at least 100 times greater than RT- PCR.

Kataria *et al.* (2001) described single-tube uninterrupted RT-PCR for direct detection of IBDV in clinical samples by amplifying the hypervariable region of the VP2 gene sequence. The VP2 gene of IBDV was amplified to generate the products of 643 and 500 bp for all the five isolates of IBDV. Kataria *et al.* (2004) also performed RT-PCR on seventeen bursal samples suspected for IBD which were collected from different parts of India. Amplicon of 643 bp was generated in 14 samples.

Chen *et al.* (1998) amplified VP2 region of IBDV genome from nine Mainland Chinese strains by RT/nested PCR using two sets of primers. The outer primers (P1 and P2) generated an amplicon of 643 bp and the inner primers (P3: 5' – GCC CAG AGT CTA CAC CAT AAC TGA – 3'; P4: 5' – GCG ACC GTA ACG ACA GAT CC – 3') annealed to the complement of bases 611- 630 and bases 1081–1100 of ORFA–1, respectively.

In the present study RT-PCR has been found to be a very sensitive test for diagnosis of IBD in field outbreaks.

CHAPTER-V

Summary, Conclusions and Suggestions for Future Research Work

CHAPTER-V
SUMMARY, CONCLUSIONS AND SUGGESTIONS FOR FUTURE RESEARCH WORK

5.1 SUMMARY

Infectious bursal disease (IBD), an economically important infectious viral disease of poultry, is caused by infectious bursal disease virus (IBDV) belonging to *Avibirnavirus* genus of *Birnaviridae* family. The disease causes considerable morbidity and mortality mainly by immunosuppression. Emergence of very virulent IBDV (vvIBDV) strains in different parts of the world including India during the last couple of decades, have demanded further research efforts in understanding the added complexicity of the disease process and the means to diagnose and control it. Present study was undertaken to know the Prevalence, Pathology and Molecular Diagnosis of Infectious Bursal Disease in Broilers.

In the prevalence study data were collected for the period of 8 months from February 2012 to October 2012 from Durg, Rajnandgaon and Raipur districts of Chhattisgarh. The broiler poultry flocks up to 3 weeks to 6 weeks were found affected with mortality 12%, 10% and 8% in the Durg, Rajnandgaon and Raipur districts respectively. In the present study it has been observed that IBD not only produced huge mortality, but it also causes immunosuppression and made the birds susceptible to other diseases leading to higher economic losses in commercial farms.

Commercial broiler farms in Durg, Rajnandgaon and Raipur district of Chhattisgarh formed the basis of the present study. As per the history recorded from the farmer, the affected broilers exhibited anorexia, marked depression, yellowish white diarrhea and

soiled vent feathers with prostration. Some affected birds showed the signs of vent pecking, trembling, lameness and staggering gait.

Most of the birds suspected to have died of IBD showed gross lesions consisted of petechial and ecchymotic haemorrhages in the leg and breast muscles, haemorrhages at the Junction between gizzard and proventriculus, congetion and haemorrhages on the serosal and mucosal surfaces of the bursa, swollen and mottled or severely atrophied spleen and swelling and paleness of kidney, Microscopic examination revealed severe depletion of lymphocytes in the bursa of Fabricius, spleen and thymus. Degenerative and necrotic changes along with haemorrhage in the kidney and necrosis of hepatocytes in the liver were observed in the IBD infected birds.

There was significant ($P \leq 0.05$) increase in the hemoglobin values in all the three IBD infected flocks (Durg - 10.59 ± 0.13, Rajnandgaon - 10.37 ± 0.11 and Raipur -10.32 ± 0.16) as compared to the normal healthy broilers (9.8 ± 0.17). the PCV values was significantly ($P \leq 0.01$) increased in all the IBD infected flocks (Durg - 29.43 ± 0.39, Rajnandgaon - 29.26 ± 0.45 and Raipur - 29.02 ± 0.42) was observed in comparison to the normal healthy broilers (25.54 ± 0.95). There was non significant increase in the TEC values in all the IBD infected flocks (Durg - 3.054 ± 0.05, Rajnandgaon - 3.001 ± 0.11 and Raipur - 3.011 ± 0.05) as compared to the normal healthy birds (2.85 ± 0.04). Significant ($P \leq 0.01$) increase in the TLC values in all the three farms (Durg - 28.3 ± 0.92, Rajnandgaon -26.7 ± 0.85 and Raipur - 19.9 ± 0.38) compared to the normal healthy birds (19.9 ± 0.44).

There was significant ($P \leq 0.01$) increase in the heterophil percentage in all the three flocks (Durg - 19.5 ± 1.12, Rajnandgaon - 22 ± 0.47 and Raipur - 19.6 ± 1.08) compared to the normal healthy birds (16.1±0.62). Further hematological investigation revealed significant ($P \leq 0.01$) decrease in the lymphocyte percentage in all the three farms (Durg - 64.8 ± 1.87, Rajnandgaon - 63.1 ± 1.42 and Raipur - 62.7 ± 1.40) compared to the normal

healthy birds (73.2 ± 0.89). Significant Eosinophil percentage was significantly ($P \leq 0.01$) decreased in all the IBD infected flocks (Durg - 0.8 ± 0.25, Rajnandgaon - 0.9 ± 0.23 and Raipur - 1.2 ± 0.20) was observed in comparison to the normal healthy birds (3.4±0.34). Non significant changes were observed in the basophil percentage in all the three farms (Durg - 1.7 ± 0.15, Rajnandgaon - 1.7 ± 0.15 and Raipur - 1.9 ± 0.10) as compared to the normal healthy birds (1.5 ± 0.17).

ELISA test was performed using serum samples collected from IBD infected birds from each flock showing signs suggestive of IBD from Durg, Rajnandgaon and Raipur district. The ELISA test in the present study revealed antibody titer which ranged from 3689 -7974 (mean titer 5602 ± 567.86), 4976 - 7393(mean titer 6243 ± 348.36) and 4990 – 6516 (mean titer 5522 ± 343.00) in affected broilers of Durg, Raipur and Rajnandgaon districts respectively.

To confirm IBD reverse transcriptase - polymerase chain reaction was carried out using clinical samples of bursae of Fabricius and spleens of broilers showing lesions suggestive of IBD from Durg, Rajnandgaon and Raipur district using IBDV specific primers. A total of 53 samples were used for the detection of virus. Out of 53 IBD affected samples of bursa and spleen, 45 samples produced an amplicon of 643bp indicating positive result (84.9%) for IBDV. Thus it appears that RT-PCR is a very sensitive test for the detection of IBDV.

5.2 CONCLUSIONS

Following conclusions can be drawn from the present study:

1. Mortality rate due to IBD in commercial poultry farms of Durg, Rajnandgaon and Raipur districts ranged from 8 to 12%.
2. Mortality was found to be more in the age groups of 3 to 6 weeks broilers.
3. There were significant decrease in the organ weight of bursa of Fabricius and spleen of IBD affected broiler birds.
4. Significant increase in Hb **(**g/dl**)**, PCV (%), TLC **(**10^3/µl**)** and heterophil (%) and decrease in lymphocyte (%) and eosinophil (%) were observed in all the broiler birds of the IBD affected flock.
5. Gross lesion in IBD affected broiler birds consisted of haemorrhages at the junction between gizzard and proventriculus, congestion and haemorrhages on the serosal and mucosal surfaces of the bursa, swollen and mottled or severely atrophied spleen and swelling and paleness of kidneys.
6. Histopathologically, there were severe depletion of lymphocytes in the bursa of Fabricius, spleen and thymus. Degenerative and necrotic changes along with haemorrhage in the kidney and necrosis of hepatocytes in the liver were observed.
7. ELISA test appeared to be a very sensitive test for assessing the antibody titer of IBDV.
8. RT-PCR appeared to have higher sensitivity for the detection of IBD field virus.

5.3 SUGGESTIONS FOR FUTURE RESEARCH WORK

1. Real time PCR needs to be developed for the detection and quantitation of IBDV genomes in poultry population of Chhattisgarh.
2. The amplicon size of 643bp amplified in the present study need to be cloned and sequenced to find variations in the IBDV genome present in Chhattisgarh.
3. Molecular epidemiological information of IBDV in Chhattisgarh needs to be investigated to formulate a vaccination strategy to control the disease.

References, Appendix I

REFERENCES

Aricibasi, M. 2010 Comparison of the pathogenesis of infectious bursal disease virus (IBDV) in genetically different chickens after infection with virus strains of different virulence. Clinic of Poultry University of Veterinary Medicine Hannover pp: 8–10.

Ajinkya, S.M., Survashe, B.D. and Sardeshpande, P.D. 1980. Breakdown in immunity to Ranikhet disease with infectious bursal disease in broiler chicks. Indian Vet. J. **57**: 265-269.

Anjum, A.D., Hasran, S. and Arbi, G.S. 1993. Infectious bursal disease in chickens in Pakistan. Pak. Vet. J., **13**: 54-58.

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

Azad, A. A., Jagadish, M. N., Brown, M. A. and Hudson. P. J. 1987. Deletion mapping and expression in *Escherichia coli* of the large genomic segment of a birnavirus. J. Virol., **161**: 145-152.

Banda, A., Villegas, P., El, Attrache, J. and Estevez, C. 2001. Molecular characterization of seven field isolates of infectious bursal disease virus obtained from commercial broiler chickens. Avian Dis., **45**: 620-630.

Banda, A. and Villegas, P. 2004. Genetic characterization of very virulent infectious bursal disease viruses from Latin America. Avian Dis. **48**: 540–549.

Balachandran, C., Muralimanohar, B., Sundararaj, A.T. and Thanickachalan, M. 1991. Infectious bursal disease and its association with gangrenous dermatitis in Tamil Nadu. Indian Vet. J. **68**: 718-720.

Bais, M.V., Tiwari, A.K., Kataria, R.S., Indervesh and Das, S.K. 2003. RT-PCR amplification and cloning of both genome segments of an Indian infectious bursal disease virus. Indian J. Poult. Sci. **38**: 1-5.

Baxendale, W. 2002. Birnaviridae. In: Poultry Diseases, 5th Edition, W.B. Saunders. London, pp. 320-324.

Becht, H., Muller, H. & Muller, H. K. 1988. Comparative studies on structural and antigenic properties of two serotypes of infectious bursal disease virus. J. Virol. **69**: 631-640.

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

Barman, N.N., Roychoudhury, P. and Dutta, T.C. 2003. Double antibody sandwich ELISA for detection of infectious bursal disease virus. Indian Vet. J. **80**: 1209-1211

Campbell T.W. and Coles, E.H., 1986. Avian clinical pathology. In: Veterinary clinical pathology. Philadelphia, PA, USA, WB Saunders, pp. 289-290.

Cavanagh, D. 2001. Innovation and discovery: the application of nucleicacid-based technology to avian virus detection and characterization. Avian Pathol. **30**: 581-598.

Chen, H.Y., Zhou, Q., Zhang, M. F. and Giambrone, J.J. 1998. Sequence analysis of the VP2 hypervariable region of nine infectious bursal disease virus isolates from mainland China. Avian Dis., **42**: 762-769.

Chai, Y.F., Christensen, N.H., Wilks, C.R. and Meers, J. 2001. Characterisation of New Zealand isolates of infectious bursal disease virus. Arch. Virol. **146**: 1571-1580.

Cheville, N. F. 1967. Studies on the pathogenesis of Gumboro disease in the bursa of fabricius, spleen and thymus of the chicken. Am. J. Pathol. **51**: 527-551.

Chineme, C.N., and Cho Y. 1984. Clinicopathological and morphological changes in chickens experimentally infected with infectious bursal disease virus. Trop. Vet., **2**: 218-224.

Chowdhury, E.H., Islam M.R., Das, P.M., Dewan, M.L. and Khan, M.S.R. 1996. Acute infectious bursal disease in chickens Pathological observation and virus isolation. A.J.A.S. **9**: 465- 469.

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

Culling, C. F. A. 1963. Handbook of histopathological and histochemical techniques. 3rd Edn., Butter Worths and Co. Ltd., London.

Dash, B.B., Verma, K.C. and Kataria, J.M. 1991. Comparison of some serological tests for detection of IBD virus infection in chicken. Indian J. Poult. Sci., 26: 160-165.

De Wit, J. J., Heijmans, J. F. Mekkes, D. R. and Van Loon, W. M. 2001. Validation of five commercially available ELISAs for the detection of antibodies

against infectious bursal disease virus (serotype 1). Avian Pathol., **30**: 543-549.

Dohms, J. E., Lee, K. P., Rosenberger, J. K. and Metz, A. L. 1988. Plasma cell quantitation in the gland of Harder during infectious bursal disease virus infection of 3-week-old broiler chickens. Avian Dis., **32**: 624-31.

Dobos, P., Hill, B. J., Hallet, R., Kells, D. T. C., Becht, H. and Teninges, D. T. 1979. Biophysical and biochemical characterization of animal viruses with bisegmented double-stranded RNA genomes. J. Virol., **32**: 593-605.

Dongaonkar, V.D. and Rao, K.N.P. 1979. Serological prevalence of infectious bursal disease virus in Madhya Pradesh and Maharashtra. Indian Vet. J., **56**: 811-815.

Dormitorio, T.V., Giambrone, J. J. and Duck, L.W. 1997. Sequence comparisons of the variable VP2 region of eight infectious bursal disease virus isolates. Avian Dis., **41**: 36-44.

Elankumaran, S., Heckert, R.A. and Moura, L. 2002. Pathogenesis and tissue distribution of a variant strain of infectious bursal disease virus in commercial broiler chickens. Avian Dis., **46**: 169-176.

Eterradossi, N., Picault, J. P., Drouin, P., Guittle, M., L'Hospitalier, R. and Bennejean, G. 1992. Pathogenicity and preliminary antigenic characterization of six infectious bursal disease virus strains isolated in France from acute outbreaks. J. Vet. Med., **39**: 683-691.

Eterradossi, N. and Saif, Y. M. 2008. Infectious Bursal Disease. In Y. M. Saif, A. M. Fadly, J. R. Glisson, L. R. McDougald, L. K. Nolan, and D. E. Swayne

(eds.). Diseases of Poultry, 12th ed. Blackwell Publishing Professional: Ames, IA, 185-208.

Fahey, K. J., Erny, K. and Crooks, J. 1989. A conformational immunogen on VP2 of infectious bursal disease virus that induces virus-neutralizing antibodies that passively protect chickens. J. General Virol., **70**: 1473-1481.

Gill, B.S., Mangat, A.P.S. and Kwatra, M.S. 1988. An outbreak of Infectious bursal disease in Punjab. Poultry Guide, 73-75.

Hanson, B. S. 1962. Post- mortem lesions diagnostic of certain poultry lesions. Vet. Rec., **80**: 109-119.

Homer, B. L., Butcher, G. D., Miles, R. D., Rossi, A. F. 1992. Subclinical infectious bursal disease in an integrated broiler production operation Journal of Veterinary Diagnostic Investigation .

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

Hirai, K., Kato, N., Fujiura, A. and Shimakura, S. 1979. Further morphological characterization and structural proteins of infectious bursal disease virus. J. Virol., **32**: 323-328.

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

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

Jackwood, D. J., Hanes, G. and Miller, S. H. 1996. Infectious bursal disease viral RNA amplification using RT/PCR from bursa tissue following phenol: chloroform inactivation of the virus. Avian Dis., **40**: 457-460.

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

Jackwood, D. J., Sommer-wagner, S. E., Stoute, S.T., Woolcock, P.R., Crossley, B.M., Hietala, S.H. and Charlton, B.R. 2009. Characteristics of a very virulent infectious bursal disease virus from California. Avian Dis., **53**: 592-600.

Jackwood, D.J. and Jackwood, R.J. 1997. Molecular identification of infectious bursal disease virus strains. Avian Dis., **41**: 97-104.

Jackwood, D.J. and Jackwood, R.J. 1994. Infectious bursal disease viruses: molecular differentiation of antigenic subtypes among serotype 1 viruses. Avian Dis. **38**: 531-537.

Jackwood, D.J. and Sommer, S.E. 1997. Restriction fragment length polymorphisms in the VP2 gene of infectious bursal disease viruses. Avian Dis. **41:** 627-637.

Jackwood, D. J. and Sommer, S. E.. 2002. Identification of infectious bursal disease virus quasispecies in commercial vaccines and field isolates of this double-stranded RNA virus. J. Virol., **304**: 105-113.

Jagadish, M. N., Staton, V. J. Hudson, P. J. and Azad, A. A. 1988. Birnavirus precursor polyprotein is processed in *Escherichia coli* by its own virus-encoded polypeptide. J. Virol., **62**: 1084-1087.

Jaisankar, S. Dinakaran, A.M. and Karunakaran, K. 2003. Retrospective studies on the pattern of viral diseases in poultry in Namakkal. Indian J. Poult. Sci., **38**: 142-144.

Jayaramiah, B. and Mallick, B.B. 1975. Serological survey of infectious bursal disease virus in Gujarat State. Indian Vet. J., **52**: 673-676.

Jain, N. C. 1986. *Haematological techniques-In Sclams Veterinary Haematology*. 4thEdn., Lea and Febingerr. Philadelphia. pp. 20-86

Jordan, F.T.W. and Pattison, M. 2001. *In* Poultry Diseases, 5th, WB Saunders**.**

Kataria, R. S., Tiwari, S. K., Bandyopadhyay, J. Kataria, M. and Butchaiah, G. 1998. Detection of infectious bursal disease virus of poultry in clinical samples by RT-PCR. Biochem. Mol. Biol. Int., **45**: 315-322.

Kataria, R.S., Tiwari, A.K., Butchaiah, G. and Kataria, J.M. 1999. Purification of infectious bursal disease virus RNA and its confirmation by Reverse transcription-polymerase chain reaction (RT-PCR). Indian J. Virol., **15**: 121-123.

Kataria, R.S., Tiwari, A.K., Butchaiah, G. and Kataria, J.M. 2000. Development of probes for differentiation of infectious bursal disease virus strains of various virulence by dot-blot hybridization. Acta Virol. **44**: 259-263.

Kataria, R.S., Tiwari, A.K., Butchaiah, G., Kataria, J.M. and Skinner, M.A. 2001. Sequence analysis of the VP2 gene hypervariable region of infectious bursal disease viruses from India. Avian Pathol., **30**: 501-507.

Kataria, R.S., Tiwari, A.K., Butchaiah, G. and Das, S.K. 2004. Diagnosis of infectious bursal disease by RT-PCR. Indian Vet. J. **81**: 12-15.

Kibenge, F. S., D. J. Jackwood and Mercado, C. C.1990. Nucleotide sequence analysis of genome segment A of infectious bursal disease virus. J. Gen. Virol., **71**(3): 569-77.

Kibenge, F. S. B. and V. Dhama. 1997. Evidence that virion associated VP1 of avibirnaviruses contains viral RNA sequences. *Archives of Virology* 142:1227–1236.

Kibenge, F.S. Dhillon, A.S. and Russell, R.G. 1988. Biochemistry and immunology of infectious bursal disease virus. J. Gen. Virol., **69**: 1757-1775.

Kim, H.R., Kwon, Y.K., Bae, Y.C., Oem, J.K, and Lee, O.S. 2010. Genetic characteristics of virion protein 2 genes of infectious bursal disease viruses isolated from commercial chickens with clinical disease in South Korea. Poult. Sci., 1642-1689.

Kosters, J., Betch H., and Rudolph, R. 1972. Properties of infectious bursal agent of chicken (IBA). Med. Microb. Immun., **157**: 291-298.

Landgraf, H.; Vielitz, E. and Kirsch, R. 1967. Occurrence of an infectious disease affecting the bursa of Fabricius (Gumboro disease). Vet. Bull. **37:** 2293.

Lee, L. H., Ting, L. J. Shien, J. H. and Shieh, H. K. 1994. Single-tube, noninterrupted reverse transcription-PCR for detection of
infectious bursal disease virus. J. Clin Microbiol., **32**: 1268-1272.

Letzel, T., Coulibali, F., Rey, F., Delmas, B., Jagt, E., van Loon, A. and Mundt, E. 2007. Molecular and structural bases for the antigenicity of VP2 of infectious bursal disease virus. J. Virol. **81**:12827-12835.

Lin, T.L., Wu, C.C., Rosenberger, J.K. and Saif, Y.M. 1994. Rapid differentiation of infectious bursal disease virus serotypes by polymerase chain reaction. J. Vet. Diagn. Invest**. 6**: 100-102.

Lin, Z., Kato, A., Otaki, Y., Nakamura, T., Sasmaz, E. and Ueda, S. 1993. Sequence comparisons of a highly virulent infectious bursal disease virus prevalent in Japan. Avian Dis. **37:** 315–323.

Liu, H. J., Giambrone, J.J. and Dormitorio, T. 1994. Detection of genetic variations in serotype I isolates of infectious bursal disease virus using polymerase chain reaction and restriction endonuclease analysis. J. virol. Meth., **48**: 281-291.

Liu, X., Giambrone, J. J. and Dormitorio, T. 1998. Simplified sample processing combined with a sensitive nested polymerase chain reaction assay for detection of infectious bursal disease virus in the bursa of Fabricus. Avian Dis. **42:** 480-485.

Liu, H.J. 2000. Tissue print hybridization and reverse transcriptase PCR in the detection of infectious bursal disease viruses in bursal tissues. Res. Vet. Sci., **68**: 99-101.

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

Lukert, P. D. and. Saif, Y. M. 1997. Infectious bursal disease virus. *Diseases of Poultry*, 10th Ed. Iowa State University Press, Ames, IA, 721-738.

Lukert, P. D., and Saif, Y. M. Infectious bursal disease. 2003. In: Diseases of Poultry, 11th ed. Y. M. Saif, H.J.Barns, A.M. Fadly, J.R. Glisson, L.R. McDougald and and D. E. Swayne, eds. Iowa State Press, Ames, IA. pp. 161-179.

Lukert, P.D., Saif, Y.M., 2004. Infectious bursal disease, in: Saif, Y.M., Barnes, H.J., Fadly

Majee, S.B., Darban, G.S., Sherikar, A.A., Das, A.M. and Sahare, A.M. 2003. Isolation and identification of Newcastle disease virus and infectious bursal

disease virus from Konkan region of Maharastra. Indian Vet. J., **80**: 601-603.

Mangala Gowri, A., Jaykumar, R., Kumaran, K. and Nachimuthu, K. 1997. Evaluation of diagnostic test for the detection of infectious bursal disease virus antigen. Indian Vet. J., **74**: 737-739.

McFerran, J.B., McFerran, M.S., McKillop, E.R., Conner, T.J., McCracken, R.M., Collins, D.S., Allan, G.M. 1980. Isolation and serological studies with infectious bursal disease virus from fowl, turkeys, and ducks: demonstration of a second serotype. Avian Pathol., 9: 384-492.

Mohanty, G.C., Pandey, A.P. and Rajya, B.S. 1971. Infectious bursal disease in chicken. Curr. Sci., **40**: 181-184.

Mondal, M., Ghosh, R.C., Mandal, A.K. and Banerjee A. 2008. Histopathology changes in IBD infected layer chicken. Indian Journal of Veterinary Anatomy **20** : 58 - 59

Mittal, D., Jindal, N., Gupta, S.L., Kataria, R.S. and Tiwari. A.K. 2005. Detection of Infectious Bursal Disease Virus in Field Outbreaks in Broiler Chickens by Reverse Transcription-Polymerase Chain Reaction International Journal of Poultry Science. **4** (4): 239-243.

Muller, A. and Nitschke, R. 1987. Molecular weight determination of the two segments of double-stranded RNA of infectious bursal disease virus, a member of the birnavirus group. Medical Microbiol. Immunol., **176**: 113 121.

Muller, H., Islam, M.R. and Raue, R. 2003. Research on infectious bursal disease--the past, the present and the future. Vet. Microbiol., **97**: 153- 165.

Nachimuthu, K., Raj, G.D., Thangavelu, A., Koteeswaran, A., Palaniswamy, K.S. and Venugopalan, A.T. 1993. Seroprevalence of infectious bursal disease in Tamil Nadu. Indian Vet. J., **70**: 100-103.

Nachimuthu, K, Raj, D.G. Thangavelu, A. and Venkatesan, R.A. 1995. Reverse passive haemagglutination test in the diagnosis of infectious bursal disease. Trop. Anim. Health Prod., **27**: 43-46.

Natt, M.P. and Herick, C.A. 1954. New blood diluents for counting erythrocytes and leucocytes of chickens. Poult. Sci., **31:** 735-738.

Nambiar, K.T.K. 1960. Studies on haematology of the domestic fowl. M.V.Sc thesis submitted to the University of Madras.

Nick, H. D. Cursiefen, and Becht, H. 1976. Structural and growth characteristics of infectious bursal disease virus. J. Virol., **18**: 227-234.

Office International Des Epizooties 2004. Avian Diseases in List B: Infectious Bursal Disease (Gumboro disease). In: Manual of diagnostic tests and vaccines for terrestrial animals (mammals, birds and bees). 5th Ed. Office International Des Epizooties, Paris, France, 817-832.

OIE Terrestrial Manual 2008 Chapter 2.3.12. - Infectious bursal disease (Gumboro disease) pp -549-565.

Oladele O.A., Adene D.F., Obi T.U., Nottidge H.O., Aiyedun A.I., 2005. Sequential Hematological Study of Experimental Infectious Bursal Disease Virus Infection in Chickens,Turkeys and Ducks. Pathologie Infectieuse Revue Élev. Méd. vét. Pays trop., , **58** (4) : 211-215

Parthiban, M., Anbalgan, S., Thiagarajan, V. and Mohan, A.C. 2003. Detection of cell culture adapted and gradient purified IBD virus by various assays. Indian J. Anim. Sci., **73**: 633-634.

Parthiban, M. and Thiagarajan, V. 2000. Application of polymerase chain reaction for early detection of infectious bursal disease virus infection. Indian J. Anim. Sci. 70: 1201-1203.

Parthiban, M., Thiagarajan, V. and Ramadass, P. 2000. Application of nonradioactive hybridization assay in detection of infectious bursal disease (IBD). Indian J. Anim. Sci., **70**: 919-920.

Petek, M. Aprilei, P.N. and Cancellotti, F. 1973. Biological and physicochemical properties of infectious bursal disease virus (IBDV). Avian Pathol., **2**: 135-152.

Pitcovski, J., Goldberg, D., Levi, B.Z., Di-Castro, D., Azriel, A., Krispel, S., Maray, T. and Shaaltiel, Y. 1998. Coding region of segment A sequence of a very virulent isolate of IBDV – comparison with isolates from different countries and virulence. Avian Dis., **42**: 497-506.

Qian, B. and Kibenge, E.S.B. 1994. J. Viral. Meth., **47:** 237 – 242.

Rosenberger, J. K., and. Cloud, S. S. 1986. Isolation and characterization of variant infectious bursal disease viruses. J. Am. Vet. Med. Assoc., 189:357.

Rosenberger, J. K., Cloud, S. S., Gelb, J., Odor E. and Dohms, S. E. 1985. Sentinel birds survey of Delmarva broiler flocks. In: Proceedings of the 20th National Meeting on Poultry Health and Condemnation. Ocean City, MD., 94-101.

Sah, R.L., Kataria, J.M., Arya, S.C. and Verma, K.C. 1995. Outbreaks of acute infectious bursal disease virus causing high mortality in chickens. Indian J. Comp. Microbiol. Immunol. Infect. Dis., **16**: 7-13.

Sainsbury, D. 1992. Poultry Health and Management, 3rd, Blackwell Scientific Publications.

Saif, E.M., Aly, M., Aziz, E. and Mohamed, P.M. 2000 Epidemiological studies on gumboro disease in Upper Egypt, Assiut. Vet. Med., **42**: 223 – 241.

Schnitzler, D., Bernstein, F., Muller, H. and Becht. H. 1993. The genetic basis for the antigenicity of the VP2 protein of the infectious bursal disease virus. J. Gen. Virol., **74** (8): 1563-1571.

Sellers, H. S., Villegas, P.N., Seal, B. S. and Jackwood, D.J. 1999. Antigenic and molecular characterization of three infectious bursal diseas virus field isolates. Avian Dis., **43**: 198-206.

Sivaseelan, S. and Balachandran, C. 2003. Pathology of naturally occurring infectious bursal disease in chicken. Cheiron, **32**: 60-63.

Skeeles J.K., Lukert P.D., De buysscher, E.V., Fletcher O.J. and Brown J., 1979. Infectious bursal disease virus infections. I. Complement and virus-neutralizing antibody response following infection of susceptible chickens. Avian Dis., **23**: 95-106.

Skeeles, J.K., Slavik, M.F., Beasley J.N., Brown, A.H., Meinecke, C.F., Maruca, S., Welch, S., 1980. An age-related coagulation disorder associated with experimental infection with infectious bursal disease virus. Am. J. vet. Res., **41**: 1458-1461.

Snedecor, G. W. and Cochran, W. G. 1968. Statistical methods. 8th Edn. Iowa State University Press, Ames, Iowa.

Snyder, D.B., Vakharia, V.N. and Savage, P.K. 1992. Naturally occurring-neutralizing monoclonal antibody escape variants define the epidemiology of

infectious bursal disease viruses in the United States. Arch. Virol., **127**: 89-101.

Stram, Y., Meir, R., Molad, T., Blumenkranz, R., Malkinson, M. and Weisman, Y. 1994. Applications of the polymerase chain reaction to detect infectious bursal disease virus in naturally infected chickens. Avian Dis., **38**: 879-884.

Tham, K.M., Young, L.M. and Moon, C.D. 1995. Detection of infectious bursal disease virus by reverse transcription-polymerase chain reaction amplification of the virus segment A gene. J. Virol., **53**: 201-212.

Toroghi, R., Kataria, J.M., Verma, K.C., Senthil, K.N. and Dhama, K. 2001. Susceptibility of chicken embryo fibroblast (CEF) and BGM-70 cells for isolation of the infectious bursal disease virus (IBDV). Indian J. Comp. Microbiol. Immunol. Infect. Dis., **22**: 29-32.

Vakharia, V. N., Ahmed, B. and He, J. 1992. Infectious bursal disease virus structural proteins expressed in a baculovirus recombinant confer protection in chickens. J. Gen. Virol., **74**: 1201-1206.

Van den berg, T.P. and Meulemans, G. 1991. Acute infectious bursal disease in poultry: Protection afforded by maternally derived antibodies and interference with live vaccination. Avian Pathol., **20**: 409-421.

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

Vindevogel, H., Gouffaux, M., Meulemans, G., Duchatel, J.P. and Halen P. 1976. Maladie de Gumboro : distribution etpersistance du vims chez le poussin inoculé. Études sur latransmission de la maladie. Avian Pathol., **5**: 31-38.

Viswas, K.N., Muniyappa, L., Suryanarayana, V.V.S. and Byregowda, S.M. 2002. Nucleotide sequence analysis of variable region of VP2 gene of two infectious bursal diseases virus isolates from commercial poultry farms. Acta Virologica, **46**: 95-101.

Verma, K. C., A. S. Panisup, G. C. Mohanty, B. R. Reddy (1981): Infectious bursal disease (Gumboro disease) and associated conditions in poultry flocks of Andhra Pradesh. Indian J. Poult. Sci. 16, 385-392.

Weiss, E. and Kaufer-weiss, I. 1994. Pathology and pathogenesis of infectious bursal disease. In: Proc. International symposium on infectious bursal disease and chicken infectious anaemia, Rauischholzhausen, Germany, 21-24.

Yamaguchi, T., Setiyono, A., Kobayashi, M., Takigami, S., Fukushi, H. and Hirai, K. 2000. Infectious bursal disease live vaccines: changes in the virus population during serial passage in chickens and chicken embryo fibroblast cells. Avian Dis. **44**: 284-290.

Zahoor, M.A. Abubakar, M. Naim, S. Khan, Q.M. and Arshed, M.J. Incidence and Molecular Characterization of Infectious Bursal Disease Virus in Commercial Broilers in Pakistan. I.J.A.V.M.S. **4**: 75-80

Appendix – I

Natt and Herrick diluting fluid:

NaCl	3.88g
Na_2SO_4	2.50g
$Na_2HPO_4,12H_2O$	2.91g
KH_2PO_4	0.25g
Formaline (37%)	7.50ml
Methyl violet (2B)	0.10g
Distilled water to make	1000ml.

10% Neutral Buffered Formalin:

For 1 liter	
Formalin (40%)	100 ml
Sodium phosphate monobasic	4.0 g
Sodium phosphate dibasic	6.5 g
Distilled Water	900 ml

ABSTRACT

STUDIES ON PREVALENCE, PATHOLOGY AND MOLECULAR DIAGNOSIS OF INFECTIOUS BURSAL DISEASE IN BROILERS

Infectious bursal disease (IBD) is a highly contagious disease of young chickens caused by infectious bursal disease virus (IBDV), characterized by immunosuppression and mortality generally at 3-6 weeks of age. It has contributed significantly in overall losses to poultry industry because of increased mortality due to IBD and other diseases occurring as a result of vaccination failures due to immunosuppressive effect of the disease. The present study was undertaken to study prevalence, pathology and molecular diagnosis of IBD in broilers. Prevalence of IBD was found to be much higher in Durg (12%) district compared to Rajnandgaon (10%) and Raipur (8%) in the age group of 3 to 6 weeks. Most of the birds suspected to have died of IBD showed petechial and ecchymotic haemorrhages in the leg and breast muscles, haemorrhages at the junction between gizzard and proventriculus, congestion and haemorrhages on the serosal and mucosal surfaces of the bursa, swollen and mottled or severely atrophied spleen and swelling and paleness of kidney. Histopathologically, severe depletion of lymphocytes in the bursa of Fabricius, spleen and thymus were observed. Degenerative and necrotic changes along with haemorrhage in the kidney and necrosis of hepatocytes in the liver were observed in the IBD infected birds. Organ weight factor of bursa of Fabricius and spleen were calculated and found significant ($P<0.01$) decrease in weight of bursa of Fabricius and spleen of IBD affected birds compared to the normal birds. Ten blood samples from Durg, Rajnandgaon and Raipur district were collected and compared with ten blood samples from normal healthy birds. There were significant increase in haemoglobin (g/dl), packed cell volume (%), total leukocyte count and heterophil (%)

and significant decrease in lymphocyte (%) and eosinophil (%) in the IBD infected birds compared to healthy birds.

Serum samples were collected from the IBD affected flock for ELISA test. The ELISA test revealed antibody titer which ranged from 3689 -7974 (mean titer 5602 ± 567.86), 4976 - 7393(mean titer 6243 ± 348.36) and 4990 – 6516 (mean titer 5522 ± 343.00) in affected broilers of Durg, Raipur and Rajnandgaon district respectively. The bursae of Fabricius and spleens of broilers of Durg, Rajnandgaon and Raipur showing lesions suggestive of IBD were collected for RT-PCR. A total of 53 samples were taken for the detection of IBDV by using IBDV specific primers. Out of 53 IBD affected samples of bursa and spleen 45 samples produced an amplicon of 643bp indicating positive result (84.9%) for IBDV. Thus in the present study RT-PCR was found to be highly sensitive test for the diagnosis of IBDV in field conditions.

Dr. R. C. Ghosh

Major Advisor

Fig. 1: Effect of IBD on organ weight factor of liver and kidney in broilers

Fig. 2: Photograph of IBD infected bird showing petechial and ecchymotic haemorrhages in the leg

Fig. 3: Photograph of IBD infected bird showing petechial and ecchymotic haemorrhages in the thigh muscle

Fig.4: Photograph of IBD infected bird showing swelling of bursa of Fabricius

Fig.5: Photograph of IBD infected bird showing congestion and haemorrhages on the serosal and mucosal surfaces of bursa of Fabricius

Fig.6: Photograph of IBD infected bird showing cheesy mass in the lumen of bursa of Fabricius

Fig.7: Photograph of IBD infected bird showing swollen and mottled appearance of spleen

Fig.8: Photograph of IBD infected bird showing atrophied spleen (a) and normal spleen (b)

Fig.9: Photograph of IBD infected bird showing swollen and pale kidneys

Fig.10: Photograph of IBD infected bird showing haemorrhages at the junction between proventriculus and gizzard

Fig.11: Section of bursa of Fabricius of IBD infected bird showing severe depletion of lymphocytes and necrosis (H & E X 100)

Fig.12: Fig.11 in high power (H & E X 400)

Fig.13: Section of bursa of Fabricius of IBD infected bird showing area of lymphoid depletion along with serous exudation (H & E X 400)

Fig.14: Section of spleen of IBD infected bird showing lymphoid depletion of various degrees (H & E X 400)

Fig.15: Section of kidney of IBD infected bird showing haemorrhage in the interlobular capillaries (H & E X 400)

Fig.16: Section of kidney of IBD infected bird showing degenerative and necrotic changes of proximal and distal convoluted tubules (H & E X 400)

Fig. 17: Section of kidney of IBD infected bird showing degenerative and necrotic changes of proximal and distal convoluted tubules leading to atrophy of glomerulus (H & E X 400)

Fig. 18: Section of lung of IBD infected bird showing severe pneumonic changes like thickening of interlobular septa due to heavy infiltration with mononuclear cells (H & E X 400)

Fig. 19: Section of lung of IBD infected bird showing severe congestion leading to stage of grey hepatization (H & E X 400)

Fig. 20: Section of lung of IBD infected bird showing serofibrinous exudation in the bronchiole (H & E X 400)

Fig. 21: Section of brain of IBD infected bird showing congestion (H & E X 400)

Fig. 22: Section of brain of IBD infected bird showing oedematous changes (H & E X 400)

Fig. 23: Section of liver of IBD infected bird showing severe haemorrhage (H & E × 400)

Fig. 24: Section of liver of IBD infected bird showing haemorrhage and severe necrosis of hepatocytes (H & E × 400)

Fig. 25: Section of thymus of IBD infected bird showing severe medullary lymphocytic depletion (H & E × 100)

Fig. 26: Fig. 25 in high power (H & E × 400)

Fig. 27: Section of thymus of IBD infected bird showing severe medullary lymphocytic depletion along with haemorrhage and congetion (H & E × 400)

Fig. 28: Effect of IBD infection on mean hemoglobin values (g/dl) in broilers

Fig. 29: Effect of IBD infection on mean PCV values (%) in broilers

Fig. 30: Effect of IBD infection on mean TEC values (millions/cu.mm) in broilers

Fig. 31: Effect of IBD infection on mean TLC values (thousand/cu.mm) in broilers

Fig. 32: Effect of IBD infection on mean heterophil values (%) in broilers

Fig. 33: Effect of IBD infection on mean lymphocyte values (%) in broilers

Fig. 34: Effect of IBD infection on mean eosinophil values (%) in broilers

Fig. 35: Effect of IBD infection on mean basophil values (%) in broilers

Fig. 36: Effect of IBD infection on mean monocytes values (%) in broilers

Fig. 37: ELISA test showing positive result by detecting IBD antibody

Fig. 38: Gel electrophoresis of PCR amplified product (643bp) of IBD virus from suspected bursa and spleen sample