EVALUATION OF VARIOUS TECHNIQUES USED FOR
DIAGNOSIS OF INFECTIOUS BURSAL DISEASE AND ITS
PREVALENCE IN KHARTOUM STATE

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Preface

This work has been carried out in the Department of Microbiology, Faculty of Veterinary Medicine, University of Khartoum under supervision of Dr. Abdelmalik Ibrahim Khalafalla.
DEDICATION

To the soul of my father
To my mother,
Brothers,
Sisters and
Friends for their unlimited assistance

With my deep love
ACKNOWLEDGMENTS

Before all I should praise constantly ALLA for providing and giving me the patience and health to complete this work.

I am deeply indebted to my supervisor Dr. Abdelmalik Ibrahim Khalafalla for his close supervision, patience, valuable assistance and for the generous provision of kits and reagents for the research. I am also grateful to my colleagues and the staff of the Virology Research Laboratory; Dr. Sana Awad, Mr. Sharani Omer Musa, Mr. Abdelmonem Ramadan, Miss. Mawahib Awad and Mrs. Nadia Dafa Alla.

My thanks extended to Prof. A. A. Jameel; head Department of Pathology, Faculty of Veterinary Medicine for the help in the histopathology, Dr. Ali Eragi and to every one who directly or indirectly participated in helping me to finish this work.
SUMMARY

The aim of the research is to study the epidemiology of infectious bursal disease (IBD) in Khartoum state and to improve the diagnosis of IBD through the introduction of the Reverse transcriptase- Polymerase Chain Reaction (RT-PCR).

Epidemiological data were collected from thirty farms that showed signs of IBD, the morbidity was estimated and the daily and total mortality were recorded. The owners were interviewed about the type and the doses of the used vaccine.

Eight to ten sick birds from each visit were brought for postmortem exam. The findings were recorded and bursas of fabricius were collected aseptically in sterile bottles and some bursas were collected in neutral formalin for histopathology.

The collected bursas were homogenized and treated with antibiotics and inoculated onto chicken embryo fibroblast (CEF) cell culture and on CAMs and yolk sacs of embryonated eggs. Five passages in CEF and embryonated eggs were done.

The collected samples were investigated with the agar gel immunodiffusion (AGID) test and Counterimmunoelectrophoresis (CIEP) test to detect the IBD antigen.

RNA of the samples was extracted and RT-PCR was conducted.

Twenty two days old chicks were infected experimentally with three selected isolates from Omdurman, Khartoum and Khartoum Bahri. Morbidity and mortality were compared between the three groups.

The results of the epidemiological data showed that 70% of outbreaks occurred between 6-8 weeks of age and the mean of the mortality rate was 51%. It was noticeable that the farms which used intermediate plus (or hot) vaccines had lowered in mortality when compared with the farms that used intermediate vaccines.

The AGID was found more sensitive than CIEP since it detected 83.4% of the IBD antigen in the samples while the CIEP detected 66.7% of the samples. The RT-PCR was found to be rapid, specific and was more sensitive than both of AGID and CIEP and it detected 93.3% of the IBD antigen in samples.
ملخص الأطروحه

أجريت هذه الدراسة لمعرفة وبانية مرض إنتفاخ الجرب المغذي (القمبرو) في مزارع تربية الدواجن بولاية الخرطوم ومن ثم تحسين الاختبارات العملية لتشخيص مرض إنتفاخ الجرب عن طريق إدخال تفاعل البلمرة المتسلال كوسيلة تشخيصية للمرض بدلاً من الوسائل التقليدية.

تمت زيارة 30 مزرعة دواجن ظهرت بها أعراض المرض بولاية الخرطوم وتم تقدير معدل المراضا وتم رصد معدل النفوذ اليومي والكلي طوال فترة المرض (7 أيام) وذلك لتحديد نسبة الفوق كما تم إجراء مقابلات شخصية مع أصحاب المزارع المصابة لمعرفة نوع اللقاح المستخدم وقدر مرات تكرار اللقاح.

تم أخذ طور تشخيص من أعراض المرض وتم تشريحها بعملي تشريح المرض - قسم الأمراض – كلية الطب البيطري - جامعة الخرطوم وتم رصد ملاحظات التشريح ما بعد الموت.

تم أخذ عينات جراب فايريشص والطحال وتم أخذ عينات في فورماتين متعادل للحصين النسيجي المرضي.

تم إعداد العينات في مختبرات فحص الحيوانات ومعالجتها بالمضادات الحيوية وتم تزويدها في أجنة البيض النامية وخلايا أرومات الدواجن.

وتم تمريرها لعدد خمس تمريرات.

تم تشخيص العينات عن طريق الإنتشار في هلام الجل والترحل الكهربائي المناعي كما تم استخلاص الحمض النووي الريببي من جراب فايريشص للطيران المصابة وتم إجراء تفاعل البلمرة المتسلال.

تم إحداث عودي تجريبي في مجموعة من الدجاجات في عمر 22 يوم وذلك باستخدام ثلاثة عزلات من منطقة الخرطوم وأم درمان والخرطوم بحري وذلك للمقارنة بينها في شدة المرض ونسبة الفوق لكل عينة.

أظهرت النتاج أن حوالي 75% من الإصابات للمزارع حدثت ما بين الأسبوع السادس والثامن وتم رصد متوسط معدل الفوق كنسبة مئوية لكل المزارع التي تم زيارتها والذي تم تسجيل نسبة الفوق الكلية للفوق المتسلامعنchr 22.1% كان أقل في معدل الفوق عن تلك التي استخدمت عرات فوق المتوسطة لكلاحب كانت أقل في معدل الفوق عن تلك التي استخدمت عرات متوسطة كللاح.

بمقارنة الاختبارات التشخيصية وجد أنه لم تظهر أي تغييرات مريرية في خلايا أرومات أجنة النجاح المحكونة بالعينات المحضرة من جراب فايريشص المصابة وكذلك لم تحدث تغييرات في أجنة البيض عند مقارنتها بالبيض غير المحقون من نفس العمر.

تفاعل الإنتشار في هلام الجل أظهرت 83.4% من العينات كان موجباً بينما تفاعل الترحل الكهربائي المناعي أظهر أن 66.7% من العينات كان موجباً وبذلك يتضح أن تفاعل الإنتشار في هلام الجل الأكثر حساسية من تفاعل الترحل الكهربائي المناعي.

تفاعل البلمرة المتسلال أظهر 93.3% من العينات كان موجباً وهو بذلك أشد حساسية من الاختبارين السابقين.
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<td>AGID</td>
<td>Agar gel immunodiffusion</td>
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<td>ARV</td>
<td>Avian reo virus</td>
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<td>bp</td>
<td>Base pair</td>
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<td>cDNA</td>
<td>Complementary deoxy nucleic acid</td>
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<td>CEB</td>
<td>Chicken embryo bursa</td>
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<td>CEF</td>
<td>Chicken embryo fibroblast</td>
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<td>CEK</td>
<td>Chicken embryo kidney</td>
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<td>CIEP</td>
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<td>Gut associated lymphoid tissue</td>
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INTRODUCTION

Infectious bursal disease (IBD) is a highly contagious viral disease of young chickens that causes significant economic losses in poultry industry worldwide (Lukert et al., 1991; Mohamed et al., 1996).

The first observation of the disease was made by Cosgrove in USA at Gumboro area and was named Gumboro disease (Cosgrove, 1962). Since that time the disease has been reported in many countries of the world including the Sudan in 1982 (Shuaib et al., 1982). Infectious bursal disease virus (IBDV) is an economically important pathogen of chickens with world wide distribution.

The clinical disease often occurs between 3 to 6 weeks of age. Severe outbreaks are characterized by sudden onset of depression in susceptible flocks. IBDV is hard nonenveloped virus that belongs to Birnaviridae (Brown, 1986). The virus genome consists of two segments of double strand RNA. Based on virus neutralization test (VNT) IBDV is classified into two serotypes, serotype I and II (Jackwood, 1985). Serotype I viruses are pathogenic to chickens while serotype II viruses (isolated from turkeys) are non-pathogenic to chickens.

The first outbreak in the Sudan was observed at El Obied (North Kordofan state) in 1981 (Shuaib et al., 1982). Since that time the disease has been reported in many parts of the Sudan and become a serious problem facing the poultry industry in the Sudan (Hajer & Ismail, 1987). Recently, the IBDV has become more virulent and the picture of the disease has changed and become more severe than in early outbreaks in 1980s. In the recent years IBD become the most devasting disease of chicken in the Sudan with mortality rates that exceed 50% even in vaccinated flocks. Diagnosis of IBD was made mainly by the case history, clinical symptoms and post mortem lesions. Laboratory confirmation was achieved by Agar gel immuno diffusion test (AGID) and virus isolation in cell culture or embryonated eggs. The symptoms of IBD are confused with many viral infections like Newcastle disease and Chicken anemia virus (CAV) and this may lead to difficulties in diagnosis. The AGID is of low sensitivity and a false negative result may happen. The virus isolation is laborious, nonspecific and time consuming. These methods have disadvantages like lacking of the ability to detect low levels of IBDV in tissues;
besides the recent very virulent strains of IBDV can not be grown in cell culture (Islam, 2002).

The main objectives of this study are:

1. To study the current situation of IBD in Khartoum state in terms of disease picture, morbidity and mortality and pathogenicity of the virus to nonvaccinated chickens.

2. Improving laboratory diagnosis of IBD in Sudan through introduction of reverse transcriptase polymerase chain reaction (RT-PCR). The RT-PCR results will be compared with classical techniques of AGID, CIEP and histopathology and virus isolation.
CHAPTER I
LITERATURE REVIEW

1.1 Definition:

Infectious bursal disease (IBD), infectious bursitis or Gumboro disease is a highly contagious viral disease which is characterized by destruction of lymphoid cells in the bursa of fabricius and other lymphoid organs (Cheville, 1967).

1.2 History of Infectious bursal disease:

Cosgrove 1962 was the first one who described IBD at Gumboro area in southern Delaware, USA (Cosgrove, 1962). Cosgrove described the condition as Avian nephrosis, but in the same year, Winterfield and Hitchiner (1962) described a nephritis-nephrosis syndrome in chickens with similar renal damage from the same area in USA. They isolated two strains of infectious bronchitis virus (Holt and Gray strains) and showed that inoculation of these strains into susceptible chicks was followed by clinical signs of IBD. Winterfield and Hitchiner (1962) recognized distinct difference between these two diseases and assigned the term infectious bronchitis variant viruses to Holt and Gray strains and Infectious bursal agent to the agent associated with Gumboro disease.

Since the first outbreak of IBD in USA 1962 the disease has been reported in many countries of the world including Great Britain (Maff, 1964); Germany (Landgraf et al., 1967); France (Maire et al., 1969); Australia (Vasicek, 1979) and Iraq (Mohanty et al., 1981). The first outbreak in Africa was in Chad (Provost et al., 1972) and distributed in other countries like Nigeria (Ojo et al., 1973) and Egypt (Ayoub and Malek, 1976).
IBD was thought to affect only chicken but in the year 1979 McNultey reported the first isolation of a second serotype from naturally infected turkey in Ireland (McNulty et al., 1979).

Virus neutralization test (VNT) indicates that the turkey isolate was antigenically different from the chicken IBDV isolates. Based on VNT McNulty et al (1979) designated several IBDV isolates from chickens as serotype I and turkey isolates as serotype II. McFerran et al (1980) isolated serotype II from chickens.

In the late 1980s the severity of the disease increased and in the beginning of 1990 in USA, western Europe and parts of Southern Asia variant strains of serotype I virus emerged that are more virulent than older strains. These have been officially designed in Europe as very virulent strains (vvIBDV) and have caused mortality rates of over 50%. These viruses have been isolated from flocks vaccinated with classical strain vaccines (Murphy et al., 1999).

1.3 IBD in Sudan:

The first outbreak in the Sudan was observed at El Obied (North Kordofan state) in 1981 (Shuaib et al., 1982). Since that time the disease has been reported in many parts of the Sudan and become a serious problem facing the poultry industry in the Sudan (Hajer & Ismail, 1987). In the year 1988 an outbreak of IBD was reported in Kassala by Gaffer et al (1988) and they found that there was a seroepidemiological relationship between the virus strain of Kassala and the strain of ElObied.

The virus strains of ElObied and Kassala were isolated from chicks imported from abroad, so there was a hypothesis that the virus has been introduced to the Sudan through this imported chicks (Gaffer et al., 1988). El Hassan et al (1989) reported for the first
time the presence of IBDV antibodies in the local breeds of chickens (Baladi) in the Sudan. Khalafalla et al. (1990) reported a mild infection of IBD in broiler chicks in the Sudan. Mahasin Elnour (1998) confirmed the existence of subclinical infection of IBDV in the Sudan since IBDV antibodies were detected among non-vaccinated non-previously infected chickens by using AGID test and Counterimmunoelectrophoresis (CIE) test. Recently, the IBDV in the Sudan has become more virulent and the picture of the disease has changed and become more severe than in early outbreaks of 1980s. Nowadays Gumboro has become number one killer of chicks in commercial poultry farms in Sudan (Khalafalla, personal communication).

1.4 Virus classification:

Initially IBDV was classified as a picorna virus (Cho, 1970a; Lungen & Madux, 1972) then as a Reovirus (Petek & Mandelli, 1968; Koster et al., 1972), later as a Blue tongue- like virus (Orbivirus) (Hiari & Shimakura, 1974), However Murphy et al. (1999) confirmed that IBDV belongs to Birnaviridae which consists of three genera, Avibirnavirus, Aquabirnavirus and Entomobirnavirus. IBDV is a member of Avibirnavirus.

Two serotypes of IBDV were recognized based on virus neutralization test. Serotype I viruses are pathogenic to chickens and vary in their virulence, whereas serotypes II are pathogenic for both turkeys and chickens (Lukert and Saif, 1991). Neutralization antibodies to serotype I strain (strain OH) cannot protect chicks from challenge with a virulent serotype I (stains STC) strain (Lasher and Shane, 1994). Serotype II IBDV produces mild histological lesion in the bursa, spleen and hardervian gland of 1-day old specific pathogen free (SPF) chicks (Sivanandans et al., 1986). The serotype I viruses are antigenically heterogenous and can be divided into four groups:
Classical virulent strains, attenuated strains, antigenic variant strain and very virulent strains.

1/ Classical virulent strains:

Cause severe lymphoid necrosis and bursal inflammation in infected chicks and lead to immunodeficiency and moderate mortality. Mortality peaks in the third day post infection (PI) but death may still occur over the next 5-7 days giving total flock mortality of 20-30% in SPF chickens. Strains 52-70, STC and 002-73 belong to classical virulent strains.

2/ Attenuated strain:

Have been adapted to cell culture, chicken bursal lymphoid cells, chicken embryo kidney (CEK) and chicken embryo fibroblast (CEF) (Lukert and Saif, 1991). Cell culture adapted IBDV strains have their virulent reduced and are used as attenuated live virus vaccines. However attenuated live IBDV strains may not induce immunity in chickens in the presence of high levels of maternal antibodies because they will be neutralized.

3/Antigenic variants:

They appeared in 1984-85 and reported on Delmarva Peninsula in USA (Rosenberger et al., 1986). Antigenic variant strains are recognized by their ability to escape cross-neutralization from the antiserum against classical strains (McFerran et al.,
1980). The strains are highly cytolytic and cause rapid bursal atrophy (within 72 hours PI) with minimal antinflammatory response. There is a variety of field infections especially those of respiratory system may arise from immunosuppression caused by variant strains (Faragher et al., 1974). Some commercial companies incorporated both classical and variant IBDV vaccine strains into commercial vaccines to enhance antigenic spectrum.

4/ The very virulent strains:

Such as UK661, they spread rapidly through out Europe, including UK in the late eighties. In summer 1990 outbreaks of acute IBD with a high mortality rate occurred in broiler flocks in western Japan. The disease spread dramatically throughout the country within the following 6 months and resulted in heavy economic losses in Japan. The initial outbreaks were characterized by morbidity rate of 80% and corresponding significant mortality, attaining 25% in broilers and 60% in pullets in a 7 day period (van den Berg et al., 1991; Nunoya et al., 1992). The European very virulent strains were regarded as classical serotype I strains because antibodies against classical could neutralized the very virulent strains in virus neutralization test. The new strains caused typical lesions of IBDV and were antigenically similar to the classical European strains, which had been prevalent for some decades. The infection with these very virulent strains caused complete loss of follicular architecture. These strains could establish infection in the presence of high level of maternal antibodies that were protective against classical strains (Chettle et al., 1989) and cause up to 60-100% mortality (van den Berg et al., 1991). Genetically there is a clear difference between the very virulent strains and other strains regarding the gene VP1 (Islam et al., 2001). Probably a mutation of one amino acid in a
hyper variable region locus in the VP2, caused change of epitope and gave arise to some of the new variants arising from USA (Giambrone, 2001).

1.5 Virion properties:

The virion of IBDV is none enveloped, hexagonal in outlines 60 nm in diameter with a single shell having icosahedral symmetry. The genome consists of two molecules of linear double stranded RNA, designated A and B, 6 kbp in overall size. Segment A is about 3.2 kbp and segment B is 2.8 kbp (Murphy et al., 1999). Segment A encodes for viral proteins VP2, VP3 and VP4, which are produced by auto proteolysis of 110 KDa precursor protein from a single large open reading frame (ORF) (Hudson et al., 1986). VP2 and VP3 are the major structural proteins of the Virion. VP2 is the major host protective antigen of IBDV and contains the antigenic region responsible for the induction of neutralizing antibodies (Eterradossi et al., 1997b), whereas VP3 considered as a group specific antigen as it recognized by monoclonal antibodies directed against VP3 from strains of both serotype I and II (Becht et al., 1988). VP4 is involved in the processing of 110 KDa precursor poly protein (Jagadish et al., 1988). Also segment A encodes for putative VP5, a 17 KDa and non structural protein encoded from small ORF which has been shown to be important since it causes lyses of host cells and release of the virus. Expression of VP5 in a cell is cytotoxic. The protein accumulates in the plasma membrane and causes cell deformation prior to cell lyses (Lombardo, 1999). Segment B encoded for a 90 KDa RNA dependent RNA polymerase protein, VP1 (Azad et al., 1985).

1.6 IBDV replication:
IBDV replicate in both chicken and mammalian cells; however, highly pathogenic strains are often difficult to cultivate (Murphy et al., 1999). IBDV replicates in the cytoplasm without greatly depressing cellular RNA or protein synthesis. The viral mRNA is transcribed by a virion-associated RNA-dependent RNA polymerase (transcriptase). RNA replication is thought to be initiated independently at the ends of the segments and to proceed by strand displacement with interval terminal repeats at the end of each segment playing a role in replication (Murphy et al., 1999).

1.7 Immunosuppression:

It has been known that most of the economic losses due to IBD are based on the induction of immunosuppression in susceptible chicken flocks.

Immunosuppressed flocks have a high incidence of secondary infection, fail to respond properly to routinely used vaccines, and show poor weight gain (Rautenschlein et al., 2001a).

Several explanations are considered to show the mechanism of suppression (Rautenschlein et al., 2001a).

1\ Suppression of humoral response:

The humoral immune response represents one important branch of the immune system. B cells, which produce antibodies against infectious and non-infectious agents, are key cell populations of humoral response. The main target cell for IBDV replication are actively dividing B cells; thus infection leads to destruction of B cells in the bursa of fabricius, the primary organ of B cells development, and, to lesser degrees, in other lymphoid organs such as caecal tonsils and spleen. The destructive effect of IBDV on B
cells lead to dramatic reduction in the ability of IBDV-infected bird to produce antibodies against agent (Rautenschlein et al, 2001a).

2\ Suppression of the cell-mediated immune response:

In addition to the reducing humoral immunity, IBDV also compromises the cell-mediated immune (CMI) response in chickens. T cells are one of the important cell populations accounting for the cell-mediated immuno response. It was demonstrated that T cells are severely suppressed in their invitro proliferation response to T cell mitogens in the acute phase of IBDV. This indicates that the T cells responsiveness is reduced and the cell immune response is impaired (Rautenschlein et al, 2001a).

3\ Suppression of microphage function:

IBDV may also modulate the function of innate immune system such as phagocytic cells of the monocyte/macrophage group. Some of these cells phagocyte and destroy IBDV and support viral replication and undergo cell death.

Chicks infected early with IBDV were more susceptible to inclusion body hepatitis (Fadley et al., 1976), Coccidiosis (Anderson et al., 1977), Marek's disease (Cho, 1970b; Sharma, 1984), Hemorrhagic aplastic anemia, Infectious laryngotracheitis (ILT) and gangrenous dermatitis (Rosenberger et al., 1978), Infectious bronchitis (Pejkovski, et al.,1979), Chicken anemia agent (Yuasa et al.,1980) and Salmonella and Colibacillosis (Wyeth, 1975). Birds infected with IBDV that were later inoculated with Mycoplasma synoviae (day 14), NDV (day 28) experienced an increase incidence and greater severity of air saculitis than did chicks which were not exposed to IBDV (Giambrone, 1977). Despite the high contagious nature, the mortality from infection with classical and variant
strains of IBDV was very low; most of the mortalities were due to immunosuppression and subsequent secondary infection (Cavanagh, 1992). There was moderate suppression when chicks were infected at 7 days and negligible effects when infection was at 14 or 21 days (Farargher et al., 1974). Hirai et al (1979) demonstrated decreased humoral antibody response to other vaccines as well.

1.8 Epidemiology of IBD:

1.8.1 Clinical feature of IBD:

The incubation period of IBDV in chickens is about 2 to 3 days. The first sign in affected chickens is an acute onset of depression. Birds are disinclined to move and peck their vents. Affected chickens suffered from whitish diarrhea, anorexia, depression, ruffled feather, trembling and severe prostration (Cosgrove, 1962). Feed intake is depressed but water consumptions are elevated. The chicks also become dehydrated and have a subnormal temperature. Affected flocks show depression for 5 to 7 days during which mortality rises rapidly for the first two days and then declines as clinical normality returns (Parkhurst, 1964). Bursa of fabricius, the primary target of the virus, is the first internal organ that shows lesions within 24 hours after infection (Helmboldt et al., 1964). The infected bursa has a gelatinous yellowish transudate covering serosal surface. The transudate disappeared as the bursa returns to its normal size and became gray in colour during the period of atrophy. At day 3 the bursa increases in size and weight because of edema and hyperemia. At day 4 the size of bursa is usually doubled its normal size and then it begins to recede in size. At day 8 the weight of bursa is usually one-third of its original weight.
1.8.2 Subclinical IBD:

Subclinical IBD occurs when chickens are exposed to IBDV during the first two weeks post hatch and have sufficient maternal antibody at time of infection to prevent clinical disease but not viral replication in the bursa. Subclinical IBD is characterized by bursal atrophy, immunosuppression and resultant increased susceptibility to secondary infections (such as *E. coli*). In this case there is no peak mortality as evidenced with clinical IBD. Secondary infections in broilers, mainly by *E. coli*, result in a continuous above standard daily mortality and poorer feed conversions (McIlory, 1994). Due to immunosuppression there can be a poor response to subsequent vaccinations.

1.8.3 Spread and transmission of IBDV:

Infectious bursal disease is highly contagious. IBDV is stable at pH 3 to pH 9 and can survive 60°C for 60 minute (Murphy *et al.*, 1999). Due to the hardy nature of the virus it persists in the environment of the poultry house, infections are thus potentially carried over from one cycle to the next.

IBDV is excreted in the feces of the infected birds for 2-14 days; it is highly contagious and transmission occurs directly through contact and oral uptake (Murphy *et al.*, 1999). The virus can be transmitted from infected pens to others by contaminated tools, equipment, feed or water, boots or clothes of labors and all peoples who move from farm to others. The virus is very stable and persists in the environment of poultry house for at least 4 months. Water, feed and dropping taken from infected pens are infectious for 2 months (Lukert and Saif, 1997). Usual cleaning and disinfection measures often do
not lead to elimination of the virus from contaminated premises, hence indirect transmission via contaminated feed, water, dust, litter and clothing or mechanical spread through insect may reintroduced the virus. The lesser meal worm _Alphitobius diapernus_ when fed as a ground suspension was infectious to susceptible chickens for 8 weeks after an outbreak (Lukert and Saif., 1991). IBDV was demonstrated in tissue samples of rat’s coats dead in a house that has a history of IBD (Lasher and Shane., 1994). Vertical transmission probably occurs via eggs (Murphy et al., 1999). Howie and Thorsen (1981) have isolated IBDV from _Aedes vexans_ mosquitoes. The Becht strain of IBDV was compared with a virus isolated from _Aedes vexans_ mosquito virus. The viruses were compared with respect to cell culture host range, cellular changes resulting from viral infections, growth curves, antigenic relationship and physiochemical characteristics. The viruses were closely comparable in all these properties, and they are considered to be strains of the same virus. The virus which isolated from mosquitoes differs from the most strains of IBDV in that it is non-pathogenic for chickens (Howie and Thorsen., 1981). Torents et al (2004) have studied the possibility of transmission of IBDV by dogs. A single Beagle dog was fed chicks infected by very virulent strain of IBDV. After-ward the presence and the viability of IBDV was detected in dog feces for 2 days after initial ingestion, which indicates the excretion of IBDV in the dog feces. Comparison by molecular techniques of the administered and excreted IBDV show similar characters between the two viruses (Torents et al., 2004).

1.8.4 Pathogenicity of IBDV:

The select host of the virus is young chickens where clinical disease occurs; while in older birds the infection is essentially sub clinical. The main variables when
comparing the result of pathogenicity trails are the breed, age and the immune status of the challenged chickens, the dose and the route of inoculation of the challenged virus and the presence of contaminating agents in the inoculums.

The terms ‘variant’, ‘classical’, ‘very virulent’ have been used to qualify the IBDV strains that exhibit a different pathogenicity. Based on signs and lesions observed in two lines of white leghorn SPF chickens during an acute experimental IBD following a $10^5$ 50% egg infective dose (EID$_{50}$) challenge, North American variants IBDVs include little if not clinical signs and no mortality but marked bursal lesion. Classical IBDVs induce approximately 10-50 % mortality with typical signs and lesions whereas very virulent IBDVs induce approximately 50-100 % mortality with typical signs and lesions (Eterradossi et al., 2001). Aflatoxins have been shown to have the effect of making IBD a much more severe disease and of changing symptoms (Chang and Hamilton, 1982). Susceptibility of different breeds of chickens has been described with higher mortality rates in light than heavier breeds (Bumstead et al., 1993). The highest susceptibility to acute IBD occurs in chickens between 3-6 weeks of age (Lukert & Saif, 1997). Inoculation of these viruses in other avian species fails to induce disease. The target organ of the IBDV is the bursa of fabricius which is specific reservoir of B lymphocyte in avian species. Bursectomy can prevent illness in chicks infected with virulent virus (Hiraga et al., 1994; Murphy et al., 1999). The severity of the disease is directly related to the number of susceptible cells present in the bursa of fabricius. Therefore the highest age of susceptibility is between 3-6 weeks, when the bursa of fabricius at its maximum development. The age susceptibility is broader in the case of vvIBDV (van den Berg et al., 1991; Nunoya et al., 1992). After oral infection or inhalation, the virus replicates primary in the lymphocyte and macrophage of gut-associated tissue. Then virus travels to the bursa via blood stream, where replication occurs. By 13 hours post inoculation PI,
most follicles are positive for virus and by 16 hours PI, a second and pronounced
viraemia occurs in other organ leading to disease and death (Muller et al., 1979). When
infection occurs earlier in the life the immunosuppression properly will be permanent. In
other cases it is usually transient. Some birds may also show growth retardation after
recovery (Sainsbury, 2003). No disease occur after 15-16 weeks of age when the bursa is
regressed (Engstrom, 2003) and chickens over six weeks of age seldom are sick but
seroconvert. This age dependent disease is caused by the virus exclusive infection of only
B-lymphocytes during differentiation (Murphy et al., 1999). The pathogenicity of IBDV
may be associated with the virus antigen distribution in non-bursal lymphopoietic organs
(Tanimura et al., 1995).

1.8.5 Morbidity&Mortality:

Before 1987, in most part of the world, IBD was sub-clinical and was
satisfactory controlled by vaccination as incriminated, while highly contagious virus
caused less than 5% mortality, with indirect essentially economic losses due to
immunosuppression. But, since 1987, vaccination failures have been described in
different parts of the world. When IBDV is newly introduced into a flock, morbidity
approaches 100% and mortality may reach up to 90% (Murphy et al., 1999). Morbidity
and mortality depend on the virulence of the challenged virus, the immune status and age
of the infected birds and other factors affecting the pathogenicity of IBDV like aflatoxins
infection.

1.8.6 The highly virulent IBDVs:
In 1984/85 variant strains of IBD started to appear in USA with increased mortality even in vaccinated flocks and this new American strains were antigenically different from classical serotype I in that they produced a very rapid bursal atrophy with minimal bursal inflammation. Vaccines prepared from classical strains did not give full protection against the variant IBDV strains (Snyder, 1990). In 1987 a highly pathogenic strain (849 VB) of type I IBDV emerged in Holland and Belgium (van den Berg et al., 1991). Mortality in exposed 3-14 weeks old layer replacement pullets attained 70% and 100% in experimental infection. Gaudry (1993) reported outbreaks of vvIBDV in China and Russia in 1993 associated with 60% mortality in 10 days old Leghorn pullets. A virus responsible for outbreaks of vvIBDV in United Kingdom designated the DV86 strain was characterized by Chettle et al (1989), who confirmed that spontaneous enhancement of virulence had occurred without any major alteration in antigenic structure. Since then, vvIBDV have been isolated in many countries including Japan (Nunoya et al., 1992; Lin et al., 1993), central Europe (Savic et al., 1997), Middle East, South America (Di Fabio et al., 1999) and Asia (Cao et al., 1998).

1.9 DIAGNOSIS OF IBDV:

2.9.1 Post mortem findings:

IBD is characterized by an acute onset, relatively high morbidity in susceptible flocks. Diagnostic lesion includes muscle hemorrhages and bursal enlargement (Hansen, 1967). Pathogenomic gross lesions observed in the bursa of fabricius which show doubling in size with a yellowish gelatinous film that may surround it and some times hemorrhages may seen on the surface of it (Cho and Edgar., 1972).
1.9.2 Histopathology:

The Histopathological changes in the infected bursa of fabricius can be observed in formalin fixed paraffin embedded sections which stained by haematoxylin and eosin stain. IBDV affect primarily the lymphoid structures, cloacal bursa, spleen, thymus, harderian glands and cecal tonsils, gut associated lymphoid tissue (GALT) and head associated lymphoid tissue (HALT) (Lukert and Saif, 1997). Histopathological changes in the bursa from chicken experimentally infected with IBDV were described by Gordan & Jordan (1982); Okoye (1983) and Lang et al (1987). Infiltration of bursa by lymphocytes was the initial lesion observed followed by necrosis of the plasma and the plast cells which resulted in destruction of all lymphoid tissue in the bursa leaving reticular structure of the follicles. Phagocytosis of necrotic lymphoid cells by large pale reticular cells progressed to severe reticular hyperplasia. By 1-5 days after infection, pyknotic debris and still more reticular cells appeared replacing the rapidly vanishing lymphocytes. Later, the centre of the bursa contains necrotic debris and intrafollicular area was oedemated. Within 3-4 days post infection there is an inflammatory hypertrophy of the bursa and apoptosis of B-lymphocytes. In disease caused by some North American subtypes this development is more rapid but bursal atrophy is not inflammatory (Eterradossi, 2001). The less virulent strains can also give histological changes in other lymphoid organs such as thymus, spleen, bone marrow and cecal tonsils. The extent of thymus lesions varies with different strains of virus (Inoue et al, 1994). Sharma et al (1993) have found evidence that lesion can occur in the thymus without replication.
Large casts of homogenous material appeared in kidneys (Helmboldt & Garner, 1964). In the spleen there was necrosis of lymphoid cells (Hitchiner, 1987 and Gordan & Jordan, 1982). In the liver, infiltration of heterophils, edema and hepatic coagulative necrosis often occurred (Cho & Edgar, 1972 and Gordan & Jordan, 1982).

1.9.3 Immunofluorescence technique:

IBDV can be detected by using immunofluorescence of impression smears of bursal tissue and this technique can detected the IBDV at 24 hours PI and the virus can be detected for 3-4 days later (Murphy et al., 1999). Direct immunofluorescent on impression smear of bursa of fabricius was studied by Allan et al (1984). They found that the direct immunofluorescent was more sensitive than the electron microscope and virus isolation (Allan et al., 1984).

1.9.4 Electron Microscopy:

IBDV can be diagnosed by electron microscopy of infected bursa of fabricius (Murphy et al., 1999). The morphology of IBDV was seen in electron microscopy of infected cell-suspensions. The negative staining to see the virus if any, under the electron microscope (Srivastava et al., 1989). The bursa of fabricius and the thymus were sampled for electron-microscopic investigations. The ultrastructural changes in the infected cells consisted of pycnosis of the lymphocyte and macrophage nuclei, vacuolization of the cytoplasm and production of lipids droplets as well as of inclusion bodies of varying
density, myelin figures, multivericular bodies and other irregular structures in the cytoplasm (Savova and Bozhov, 1985).

1.9.5 Serological tests:

1.9.5.1 Agar gel immunodiffusion test (AGID):

The AGID is the most useful of serological tests for detection of specific antibodies in serum or for detecting viral antigen in bursal tissue (OIE, 2004). The test is specific because it can not give false positive results, but it can give a false negative result. The presence of IBDV antigen can be detected in the bursal tissue by AGID for 5-6 days PI (Murphy et al., 1999).

1.9.5.2 Quantitive AGID test:

The AGID can also be used to measure antibody levels by using dilution of serum in the test well and taking the titer as the highest dilution to produce a perciptin line (Cullen & Wyeth, 1975). This can be very useful for measuring maternal or vaccinal antibody and for deciding on the best time of vaccination; however this quantative AGID determination has now been largely replaced by the ELISA.

1.9.5.3 Virus Neutralization Test (VNT):

VNT is carried out in cell culture. The test is more laborious and expensive than the AGID, but is more sensitive for detecting antibody. The sensitivity is not required for routine diagnostic purpose, but may be useful for evaluating vaccine responses or for differentiation between IBDV I and II serotypes (OIE, 2004). It is difficult to use VNT in recent vvIBDV stains, because they are difficult to cultivate in cell culture.
1.9.5.4 Enzyme linked immunosorbent Assay (ELISA):

ELISAs are in use for the detection of antibodies to IBD. Coating the plates requires a purified or at least semi purified preparation of virus, necessitating special skills and techniques. Methods of preparation of reagent and application of assay were described by Marquardt and co-workers in 1980 (Marquardt et al., 1980). Antigen-capture ELISA (Ac-ELISA) had been used for the detection of IBDV antigens directly from infected tissues (Snyder et al., 1988).

1.9.5.5 The rocket immunoelectrophoresis (RIE):

The RIE test was for qualification detection and quantitative estimation of IBDV specific antigen in experimentally infected chickens and samples collected from suspected outbreaks (Raj et al., 2000). The IBDV was detected in the bursa of experimentally infected chickens up to 5 days PI by the AGID test and up to 7 days by the RIE test. Exudative bursas were found to have higher antigen content than haemorrhagic bursa and are recommended as the material of choice for diagnosis. The test could be used to quantify IBDV specific antigen in commercial killed vaccines.

1.9.5.6 Counterimmunoelectrophoreses (CIEP):

IBDV antigen was detected in the bursa of fabricius of infected birds by the use of Counterimmunoelectrophoresis technique (Durojaiye et al., 1985). The test was also suitable for detection of antibody to IBDV in sera of infected birds. Precipitin lines were visible within 30 minutes as compared 18 to 24 hours with Ouchterlony agar precipitation test.
A rapid diagnostic strip for IBD was developed based on membrane chromatography using high-affinity monoclonal antibodies directed to IBDV. The diagnostic strip has high specificity for detection of chicken IBDV antigen and recognizes a variety of virus isolates, including virulent and attenuated strain, with no cross-reactivity to other viruses, such as Newcastle disease virus, Marek’s disease virus, infectious bronchitis virus, infectious laryngotracheitis virus and egg-drop syndrome virus. The results showed that its specificity was highly consistent with the AGID. The diagnostic strip is 32 times more sensitive than AGID (Zhang et al., 2005).

1.9.6 Virus Isolation:

1.9.6.1 Embryonated Eggs:

Hitchiner (1970) demonstrated that the chorioallantoic membrane (CAM) of 9-11 days old embryos was the most sensitive rout for isolation of IBDV which could subsequently be adapted to allantoic sac and yolk sac rout of inoculation. Hitchiner (1970) observed that the most mortality of embryos occurred between the 3rd and 5th day PI. The recent very virulent strains are difficult to cultivate in embryonated eggs (Bumstead et al., 1993).

1.9.6.2 Cell culture:

The old strains or classical strains of IBDV can be isolated in chicken embryo bursa (CEB) and chicken embryo kidney (CEK) (Lukert & Davis, 1974).

The virus however didn't replicate in kidney cell until serial passage in CEB cells. The virus produces a cytopathic effect (CPE) in kidney cells in about 3-5 days (Lukert & Davis, 1974). IBDV grows in chicken embryo fibroblast and produces CPE characterized
with an appearance of round retractile cells in about 3-5 days (Sivanadan et al, 1986). IBDV isolation in cell culture is not a routine use as a diagnostic test because the virus is difficult to culture. Some field strains failed to grow on cell cultures (Bumstead et al., 1993).

Wild–type IBDV strains particularly very virulent strain do not grow in tissue culture. Comparison of genome sequence of wild-type and tissue culture adapted IBDV strains pointed to several mutations that might be responsible for invitro growth of IBDV in tissue culture (Islam, 2002).

1.9.7 Identification of IBDV by molecular techniques:

Molecular virological techniques have been developed that allow IBDV to be identified more quickly than by virus isolation (Davis &Boyle, 1990; Wu et al., 1992). The more frequently used molecular method is the reverse-transcription polymerase chain reaction (RT-PCR) (Wu et al., 1992; Lin et al., 1993). This method can detect the genome of IBDV, which is unable to grow in cell culture or embryonated eggs because it is unnecessary to grow the virus before amplification even when the virus is present in very minute quantity and has lost its infectivity (Mittal et al., 2005). RT-PCR is performed in three steps: extraction of nucleic acid from studied sample, reverse transcription (RT) of IBDV RNA into complementary DNA (cDNA), and amplification of the resulting cDNA by PCR. The two latter steps require that the user selects oligonucleotide primers that are short sequence complementary to the virus specific nucleotide sequence. Different areas of IBDV genome have been selected (OIE, 2004).

Most efforts at molecular identification have focused on the characterization of the larger segment of IBDV (segment A) and especially of the VP2 encoding region.
Several protocols have been published on characterization using restriction endonucleases (RE) of RT-PCR products. These approaches are known as RT-PCR/RE or RT-PCR-RFLP (restriction fragment length polymorphism) (Jackwood & Jackwood, 1997; Zierenberg et al., 2001).

Radiolabeled and non-radiolabeled cDNA probes have been used to detect IBDV RNA (Jackwood et al., 1989). This method was considered as rapid, sensitive and specific diagnostic procedure of IBDV infection. A multiplex PCR (mPCR) was developed and optimized for simultaneous detection and differentiation of avian reo virus (ARV), Avian adeno group I (AAV-I), Infectious bursal disease virus (IBDV) and chicken anaemia virus (CAV). The mPCR demonstrated similar sensitivity in tests using experimental fecal cloacal swabs specimens that were spiked with ARV, AAV-I, IBDV and CVA taken from SPF chickens.

This mPCR detected and differentiated various combinations of RNA/DNA templates from ARV, AAV-I, IBDV and CAV without reduction of amplification from feces. The specificity of RT-PCR for diagnosis of IBDV was further confirmed by nested PCR. Nested PCR has been considered to be more sensitive for IBDV than conventional PCR (Liu et al., 2002).

1.10 The economic effect of IBDV:

Immunosuppressive viral diseases are a great concern for the poultry industry for several years. Indeed the reemergence of IBDV in variant or highly virulent forms have been the cause of significant economic losses (Rautenschlein et al., 2001b). IBDV has an economic impact not only due to the direct losses it provokes, but also to
the indirect losses as a consequence to immunosuppression or due to interaction it might have with other factors. The direct losses are due to specific mortality, depending on the virulence and the dose of the inoculums, the age and the breed of the bird, and the presence or absence of passive immunity. More over, IBDV is also responsible for indirect losses due to acquired immunodifficiency, impaired growth and condemnation of carcasses (Shane et al., 1994). Further more the increase use of antibiotics and chemicals to fight against opportunistic (secondary) infections is a major concern of human health, if we consider the risks linked to the presence or residues in meat products, the release of residues into environment and increased antibiotic resistance (Marian, 2001).

It can be stated that there is a significant variation in body weight in Gumboro affected broilers due to the existing and imposed vaccination program under farm condition and thus the imposed vaccination program should be recommended for use in farm condition to attain better body weight (Paul et al., 2004).

The economic importance of this disease is fundamentally based on these two aspects: on one hand, the high mortality rate caused by some IBDV strains in 3-week old chickens, and even older, and on the other hand, the second clinical manifestation of the disease consisting of a prolonged immunosuppression of the birds infected at early ages (Naqi et al., 2001). The main sequelae associated to said immunosuppression are: dermatitis gangrenosa, anemia-hepatitis syndrome with inclusion bodies, E. coli infections and failures in the efficacy of other vaccinations, such as the vaccinations against the Newcastle disease and infectious bronchitis (Naqi et al., 2001).

1.11 Treatment and control of IBDV:
No known chemotherapy treatments of IBDV, but antibiotic are given to avoid or to prevent secondary infections which may occur due to immunosuppression which become a result of IBDV infection. Electrolytes and minerals are given as supportive treatment to keep the acid-base balance in equilibrium (Pettit et al., 1983).

The control of IBD and vvIBD involves many factors, such as biosecurity, disinfection, vaccination programs and understanding the epidemiology of the IBDV.
1.11.1 Biosecurity:

Basic management practices such as limited controlled site access, separate footwear and equipment for each site/house, and footbaths at the entrance to sites/houses all minimize the risk of introducing the virus. Due to the resistance of IBDV an infection on a site easily leads to an endemic situation. Hygienic measures are aimed at minimizing infection pressure. Priority is to remove contaminated litter from site as soon as possible. A structured approach is required to prevent back tracking of the virus: Dry clean is the removal and disposal of all organic material from the site (In case of earthen floors this should include removing the top 4-5 cm of soil). Wet clean is the cleaning poultry house using water at high pressure (35 – 55 Bar) to ensure removal of all organic material. It is advisable to add detergents to assist cleaning process. Disinfection is the application of suitable disinfectant to reduce infectivity of any remaining virus particles. Applying disinfectants at the correct concentration with a suitable contact time is critical. Generally products containing formaldehyde, iodophores, chlorine-releasing agents or quaternary ammonium compounds are suitable. The downtime between successive broiler flocks must be maximized (a minimum of 10 days is recommended between successive flocks).

Control of IBDV on multi-age sites is extremely challenging and requires strict control of the movement of personnel and equipment between houses. Due to high resistance of IBDV to environmental exposure hygienic measures alone are ineffective and vaccination is thus essential (Lukert and Saif, 1997).
1.11.2 Vaccination:

Vaccines form an important part of a Gumboro control strategy. The choice of vaccine to be administered depends on the type of chicken being vaccinated and the prevailing challenge situation.

1.11.2.1 Live vaccines:

They are classified into three types depending on their virulence (Lukert & Hitchner, 1984) and determination of bursa/body weight index (Mazariegos et al., 1990) in vaccinated and nonvaccinated birds:

1-Mild vaccines:

They are low in their invasiveness to the bursa and may be neutralized by maternally derived antibodies, they don’t cause lesions or immunosuppression and they are recommended to use in free or low levels of maternally immune chicks (Lukert & Hitchiner, 1984). They don’t induce significant differences in bursa/body weight index (Mazariegos et al., 1990).

2- Intermediate vaccines:

They are varying in their virulent and induce bursal atrophy and immunosuppression (Lukert & Saif, 1991). They induce temporary significant difference in vaccinated and control birds in bursa/body weight index. The bursa weight in vaccinated birds is less than half that in control unvaccinated ones (Mazariegos et al., 1990). They are used in vaccination of replacement pullets and for primary vaccination before inactivated revaccination (Lohren, 1994).
3- Hot vaccines ‘More invasive’:

They are better able to cope with the field challenge by establishing strong ‘Immunity Cushing’ in the infected areas. They showed significant in bursa/body weight index. The bursa of vaccinated birds was more than or three times less than in controls (Mazariegos et al., 1990). They are intended for use in the presence of high level of antibodies and in areas where IBD is endemic, or otherwise the vaccine may result in mortality or immunosuppression (Lukert & Saif, 1991).

1.11.2.2 Killed vaccines:

Oil adjuvant killed virus vaccines are used to boost and prolong immunity in breeder flocks that have been “primed” with live virus either in form of live vaccine or field exposure (Wyeth & Cullen, 1978).

Vaccine producer’s arguments for giving inactivated vaccines as reported by Lohren (1994) were:

1. Attaining higher level of maternal antibodies.

2. Protection of bursa of fabricius from damage and immunosuppression for the first four weeks.

3. Production of more uniform levels of maternal antibodies.

4. Broilers need not be vaccinated with live vaccines.

Effect of hydrostatic pressure on the activity of IBDV was described by Martinez et al (2000). The infectivity of IBDV decreased with the increasing of pressure and pressurizing time and may be completely lost, indicating by the tests with CEF cell culture and young chickens as model (Martinez et al., 2000). Fluorescence spectroscopy
showed obvious difference between the structures of native and pressurized IBDV, similar with the dissociation-reassociation of oligometric proteins under high pressure. The changes of pressurized IBDV were also directly observed with electron microscopy. These results suggest that the pressure-inactivated IBDV may be used as a vaccine (Martinez et al., 2000).

1.11.2.3 In ovo vaccination:

In ovo vaccination is an alternative approach to post-hatch vaccination of chickens, particularly in broilers. Vaccination of embryonation day 18 helps to close the window of susceptibility i.e. the time between vaccination and early exposure of infection agent compared with post-hatch vaccination (Negash et al., 2004). IBDV can be vaccinated in ovo and effects of maternal antibodies in vaccines used in ovo vaccination can be prevented by developing vaccines that are insensitive to maternal antibodies (Negash et al., 2004). Administering live vaccines to developing chicks in ovo proven to be fast, effective (100% of eggs receive the vaccine) and labour saving method (Giambrone et al., 1999).

1.11.2.4 Recombinant vaccines:

The concept of recombinant vaccines is to insert genes of critical immunizing epitopes of a disease agent into nonessential gene of a vector virus. Vaccination with the recombinant virus thus results in immunization against both the vector virus as well as the expressed epitopes of the disease agent.

Hung Z et al (2004) have developed a recombinant Newcastle disease virus (NDV) expressing VP2 protein of IBDV that protect against both NDV and IBD.
1.11.2.5 Vaccination programs:

The success of the vaccine and vaccination programs can be determined by profiling blood sera of vaccinated birds after vaccination. The sera can be tested by ELISA kits for measuring the level of IBDV antibodies. Another mean of determining the efficacy of vaccination program is to measure the size of the bursa as the bird age (Giambrone et al., 2001). Smaller than normal bursa could indicate that the bird is infected with IBDV and thus unable to fight the virus. However there are numerous infectious and noninfectious agents that can alter bursa size. Recommending an IBDV vaccine that will protect a flock is difficult because the antigenicity of the pathogenic field isolate causing the disease is usually unknown. The solution of this problem is the use of real-time RT-PCR to identify nucleotide sequence homology between vaccines strains and wild-type pathogenic viruses (Jackwood, 2004). Comparing sequence homology between the pathogenic and each IBDV vaccines product will identify the vaccine that matches or more closely matches the nucleotide sequence of the pathogenic virus. Since only two or three short nucleotide sequence can be compared in these assays, it is important to select regions that are important in the development of immunity to the virus (Jackwood, 2004).

Conventional IBD is controlled by immunization of parent stock followed by vaccination of progeny after maternal antibody levels have waned. Parent level birds are immunized by one or two successive doses of live, mild or intermediate strain, attenuated vaccine to prime the immune system. This is followed by inactivated oil emulsion vaccines at maturity to boost immunity and ensure transfer of protective maternal antibody to the progeny.
vvIBD can infect some chicks as young as 15 days old in spite of the presence of maternal antibodies to IBD virus. At the same time, the maternal antibody titres are too high to enable successful immunization of the young chicks with the highly attenuated live vaccine. Several vaccination strategies have been investigated in an attempt to break through the maternal antibody barrier (Wyeth & Chettle, 1990; Wyeth et al., 1992; Goddard et al., 1994).

The currently favored strategy to control vvIBD is to vaccinate the parent birds to protect their progeny as before. The progeny are then vaccinated with a killed oil emulsion vaccine at 7 days old to give a protection rate of around 85 to 90% (Wyeth & Chettle, 1990). This may be followed by live vaccine in the drinking water at around 2.5, 3.5 and 4.5 weeks of age although researchers have shown that this offers no extra protection (Wyeth & Chettle, 1990; Goddard et al., 1994). Another strategy is to vaccinate young birds with a live IBD vaccine of intermediate virulence. Addition of 5% freeze-dried IBDV-immune bovine colostrums to the diet of chicken prevented infection when housed in IBDV contaminated environments. No interference with development and an active immunity was found in chicken receiving 5% IBDV-immune colostrums in their diet and vaccinated intramuscularly with modified IBDV (Luco and Schuliz, 1980).

Serological monitoring using automated ELISA technology may be used to determine maternal antibody levels in young birds or to confirm that flocks are adequately protected. This information is useful in determining if and when to vaccinate.

CHAPTER II
MATERIALS AND METHODS
2.1 Filed visits:

Field outbreaks of IBDV were investigated in Khartoum state. Affected farms were visited and epidemiological data were collected by an interview with the owners, the morbidity was estimated and the daily mortality was followed up till the end of the disease course. A number of 8 –10 sick birds were brought to the laboratory for post mortem examination. History of vaccination against IBD, the used vaccine and the frequency of vaccine were also collected.

2.2 Post mortem examination (PM):

This was done in the PM room, Department of Pathology, Faculty of Veterinary Medicine, University of Khartoum. PM performed by using sterile scissors and forcepses. Macroscopic lesions were recorded. Bursa of fabricius and spleen were taken aseptically in sterile bottles and kept in -20°C till used. Some bursas were taken in 10% neutral formalin (Appendix 7.2) for histopathology examination.

2.3 Histopathology:

Bursas were removed from birds that showed signs of IBD and fixed directly in 10% neutral formalin (Appendix 7.2), kept at least 24 – 48 hours and then processed according to the method of Drury and Wallington (1998). Briefly, tissues were dehydrated by graded alcohol, cleared with chloroform, impregnated by changes of paraffin wax in 2 – 3 °C above the melting point (59–60 °C) and embedded in blocks. The tissues were put in vacuum system to reduce pressure, remove any residual air speed
up impregnation for 30 minutes. Thereafter, the paraffin wax- embedded tissue blocks were sectioned in 5 micron sick by rotary microtome and stained in slides with haematoxylin and eosin (H & E) and examined under light microscope.

2.4 Laboratory Diagnosis

2.4.1 Preparation and sterilization of glasswares:

Glasswares like flasks, beakers and measuring cylinders were rinsed in running tap water, brushed with special tissue culture flask soap, and left overnight in 1% HCl. Washed thoroughly by rising several times in tap and distilled water. After that they were wrapped with aluminum foil and sterilized in hot air oven at 160° C for 1 hour. The volumetric pipettes were left overnight in 3% HCl. Then they washed thoroughly by rising several times in tap and distilled water. The clean dry pipettes were cotton plugged, placed in canisters and sterilized in hot air oven at 160° C for 1 hour.

2.4.2 Preparation and sterilization of plasticwares:

Rubber liners for bottles and plastic cylinders were cleaned with detergent, washed with tap water followed by distilled water, left to dry and sterilized by autoclaving at 121° C (15lb / squire inch) for 15 minutes.

2.4.3 Preparation and sterilization of filters and filter papers:

Millipore filter papers (0.22 µ) were assembled in their appropriate holders, wrapped in aluminum foil and sterilized by autoclaving at 121° C (15lb / squire inch) for 15 minutes.
2.4.4 The AGID:

2.4.4.1 Preparation of Bursas and spleens samples for AGID:

Bursa and spleen pieces were homogenized using sterile mortars and pestles with the aid of sterile sand and normal saline (Appendix 1.1). 10% of suspensions were made and centrifuged at 1000 rpm for 10 minutes. Supernatant fluids were collected into sterile bottles and kept at -20°C till used.

2.4.4.2 IBDV antigen & IBDV Hyperimmune serum:

Standard IBDV antigen & IBDV Hyperimmune serum were kindly donated from Ditasi Company, Khartoum, in a lyophilized form (produced by Intervet International B.V. Boxmeer, Holland). Before using in AGID each vial was reconstituted in 1ml distilled water and stored at –20°C till used.

2.4.4.3 Preparation of Agar for AGID:

On a glass container 1.4 g purified agar was added to 100 ml Agar buffer (Appendix 1.7) mixed and put in a steamer for an hour and then dispensed in petri dishes in 17 ml volume per dish. The dishes were solidified by air at room temperature. After solidification 7 holes were made, 6 outer and one inner. The 7 holes make a shape of a circle. The diameter of each hole was 6 mm with 3 mm interspaces between holes.

2.4.4.4 Test procedure:-
Using a pipette 20 µL of bursal or spleen homogenates were mixed with 20 µL sodium deoxycolate, then 20 µL of the mixtures were put in the 6 around wells and 20 µL of the hyperimmune serum was put in the inner well. Then the gel was incubated in a humidified chamber at room temperature for 24 – 48 hours.

The test was read against illuminated chamber, clear precipitin lines were recorded as positive results.

2.4.5 Detection of IBDV antigens by Counterimmunoelectrophoresis:

2.4.5.1 Preparation of agar:

On a glass 1g purified agar was added to 100 ml electrophoresis buffer solution (Appendix 1.9) and the agar was dissolved by boiling for an hour.

2.4.5.2 Test procedure:

The test was performed according to Berg (1982). When the agar was melted each microscopic slide was flooded 3.5 ml of agar. After the agar was solidified two rows of wells were cut. The slides were placed in the electrophoresis chamber. Wicks cut from Whatman No.1 filter paper were used to connect the agar and the buffer. The anode oriented wells were filled with immune serum while the cathode oriented wells were filled with infected bursal homogenate of field samples. The samples were electrophorized at a constant voltage of 5 volts/cm for 45 minutes at room temperature. Reading was observed against indirect illumination. A positive reaction was shown by formation of precipitin lines between and serum wells.
2.4.6 Virus isolation trials

2.4.6.1 Preparation of Bursas and spleens samples:

Bursa and spleen pieces were homogenized using sterile mortars and pestles with the aid of sterile sand and normal saline (Appendix 1.1). 10% of suspensions were made and centrifuged at 1000 rpm for 10 minutes. Supernatant fluids were collected into sterile bottles and treated with antibiotic (1000 IU penicillin – 250 mg of streptomycin and 5000 IU of mycostatin / 1 ml of sample). The supernatant fluids were left ½ hour at 4°C and then stored at -20°C till used.

2.4.6.2 Isolation in Embryonated Eggs:

2.4.6.2.1 Fertile eggs:

These were obtained from the poultry farm of the Virology Research laboratory, Department of Microbiology, Faculty of Veterinary Medicine University of Khartoum as one day old and incubated in Funki type (1) incubator and candled daily for egg viability till used at 5-7 or 11 – 13 day old.

2.4.6.2.2 Inoculation of IBDV on the yolk sac:

Embryonated eggs of 5 – 7 days were selected and candled for viability and placed in an egg flat or other suitable holder with the large ends up. The shells were disinfected with 70% alcohol and a hole was drilled in the center of air sac. 0.2 ml of bursal sample was inoculated vertically through the hole using a 1 ml disposable syringe and needle. The inoculated eggs were candled daily and those which died before 24 hours were discarded. The eggs were incubated at 37 ºC and candled daily for 5 days.
2.4.6.2.3 Harvest of inoculated yolk sacs:

The eggs were disinfected with alcohol at the air sac area. The shells on the air sacs area were cracked off with sterile forceps. With other sterile forceps the chorioallantoic membranes (CAMs) were ruptured and the contents of the eggs were poured in sterile petri dish. By using forceps the yolk sacs were collected and ruptured to discard the yolk. Yolk sacs were homogenized in sterile mortar and pestle with normal saline and used as antigen in AGID against known positive hyperimmune serum.

2.4.6.2.4 Inoculation of the IBDV on the chorioallantoic membrane (CAM):

11–13 day old embryonated eggs were selected and candled for egg viability. A hole was made through the shell at the air sac by a pointed punch and another hole on the top of the egg just to penetrate the shell and shell membrane. The dropping of the membrane was verified in dark room by candling, and then a rubber bulb was placed over the hole in the air sac to slowly aspirate the air from the air sac by releasing pressure on the deflated bulb. The suction caused false air cell to form in the area of the second hole. To deliver the inoculums on the chorioallantoic membrane (CAM), the tip of 1ml needle was inserted just within the shell and 0.2 ml of the sample was inoculated into embryonated eggs. The eggs were incubated at 37° C, and were examined daily for 5 days. Deaths up to 48 hours were discarded.

2.4.6.2.5 Harvest of the CAMs:
After 3–5 days incubation all inoculated eggs were taken to a refrigerator for at least 1 hour. Then the shells were disinfected on the large ends of the end of the eggs with 70% alcohol, the large end of the egg was cracked off by sterile scissors and forceps. The CAMs were removed and placed in a sterile Petri dish and examined for pock lesion formation and/or any other lesions under indirect illumination.

2.4.6.3 Isolation of IBDV in cell culture:

2.4.6.3.1 Preparation of chicken embryo fibroblast cell culture (CEF):

Embryonated eggs of were obtained from the poultry farm of Virology Research Laboratory, Department of Microbiology, Faculty of Veterinary Medicine, University of Khartoum. Eggs were incubated in a Funki type (1) incubator till 9–11 day old. The incubated eggs were candled for viability and then the shell surfaces were cleaned by immersing in 70% alcohol for 5 minutes. The round shell just below air sac was cut by pairs of scissors, and the embryo was removed by placing the scissors under its head and the embryo lifted out.

The head and the limbs of the embryo were cut, viscera were removed and remains were fragmented with scissors into small fragments.

Then the fragments were washed several times with phosphate diluents (PD) (Appendix 1.3) and transferred to a trysinization flask.

The trysinization was carried out with discontinuous manner. 5ml of prewarmed trypsin versin solution (Appendix 1.4) per embryo were added and stirred slowly for 15 minutes at 37°C. After the tissue had settled out, the supernatant cells (containing single cells and small cluster of cells) were collected in container with one to two ml of calf
serum (Appendix 2.5) and kept on ice. This was repeated 3 – 4 times until only the white
fibrous tissues were left and no more cells dispense. Then the supernatant containing the
cells was centrifuged to 1000 rpm for 5 minutes. The supernatant was poured out and the
pelleted cells were resuspended by the equation of each 1 cubic centimeter cells to 100ml
of growth media and mixed by pipetting. Then the diluted suspension was distributed into
50 ml flask, 7.5 ml/flask and in 6 well plates (2 ml/well) and then incubated at 37°C.
Confluent monolayers were established within 2–3 days.

2.4.6.3.2 Inoculation of CEF monolayers

After removing the media, 50 µL of bursal homogenates were inoculated on
confluent cells grown in 6 well plates or 100 µL in 50ml tissue culture flask. Plates and
flasks were incubated for 1 hour at 37° C. Then the inoculum was aspirated and the cells
were washed with sterile PD before addition of maintenance media.

The plates and the flasks were monitored for 5 days for cytopathic effect (CPE). If
no, then passages were made with cells disturbed by freezing and thawing three times and
harvested. The harvests were reinoculated in new monolayer and monitored for any
changes in cell morphology.

2.4.7 Polymerase Chain Reaction (PCR):
2.4.7.1 RNA extraction:

TRIzol extraction procedure:
200 mg of bursal tissue was homogenized in sterile mortar & pestle using 2ml TRIzol® regent (GIBCO BRL). The homogenate was taken to 2ml eppendorf tube. Then the tube was centrifuged in a microcentrifuge at 12000xg for 10 minutes. The supernatant was removed into a fresh tube. 0.4ml of chloroform was added, the tube was mixed vigorously by hand for 15 seconds and incubated at room temperature for 2 – 3 minutes, and then the tube was centrifuged at 12000xg for 15 minutes. Following the centrifugation the mixture was separated into three phases.

The upper aqueous phase (which contains RNA) was removed into a fresh tube by pipetting. 1ml isopropyl alcohol was added to the tube containing the aqueous phase and the tube was incubated at room temperature for 10 minutes and then centrifuged at 12000xg for 10 minutes. Following centrifugation the RNA was precipitated. The supernatant was removed and the RNA pellet was washed with 2ml of 75% ethanol and mixed by vortexing before centrifugation at 7500xg for 5 minutes. After that the supernatant was dried by air for 5-10 minutes. The pellet was rehydrated with 50 µL RNase free water or RNA rehydration solution and kept at – 20°C till used.

2.4.7.2 Positive control:

The intermediate vaccine strain D78 (Intervet International -Boxmeer, Holland) was used as control positive.

RNA extraction from the vaccine:

One freeze dried ampule was dissolved in 1ml DDW and 200µL was taken for RNA extraction by TRIzol method as described before.
2.4.7.3 Control negative:

RNA prepared from normal bursa of fabricius (tested with AGID) and DDW were used as control negative.

2.4.7.4 Oligonucleotide primers and PCR condition:

2.4.7.4.1 Primers sequence and reconstitution of primers:

The sequences of oligonucleotide primers used for RT-PCR were previously selected from the highly conserved VP2 genome region derives from the published sequence (Darja et al., 2002). Primer IBDV-F (5’-ACAGGCCAGAGTCTACACCATAA-3’) and primer IBDV-R (5’-ATCCTGTGTCCTCTCTTCTCGTAGG-3’). Primers were purchased as freeze dried oligonucleotides (INVITROGEN), reconstituted in 1ml DDW and left overnight at 4°C. Working solution for each primer was prepared so as to contain 10 pmol/µl of oligonucleotides.

2.4.7.4.2 PCR procedure:

RT-PCR was carried out using a one tube RT-PCR kit (Invitrogen, USA) in a final volume of 50µl including 19 µL DDW, 25 µL 2X Reaction mix, 1µL IBDV-F, 1µL IBDV-R, 3µL template, 1µL RT/ Platinum Taq mix.

The RT-PCR was carried out with uninterrupted programme in TP3 Thermocycler (BIOMETRA, Goettingen, Germany) under the following condition: 45 minutes at 48°C for reverse transcription and 2 minutes at 94°C for transcriptase inactivation, 40 cycles of 30 second at 94°C, 1 minute at 60°C and 2 minutes at 68°C for PCR, and extension step of 7 minutes at 68°C. The final step was open time (pause) at 4°C.
2.4.7.4.3 Analysis of PCR products:

The reaction products were analyzed by electrophoresis in 1% agarose gel (Appendix 8.1) stained with ethidium bromide (1ul / 40ml agarose) (Appendix 8.5). 10ul of 100 bp DNA ladder (Invetrogen) (Appendix 8.4) was loaded in the first slot of the gel. Then 10ul of PCR products were mixed with 5ul of loading dye (Appendix 8.3) and loaded on the rest wells.

Electrophoresis was performed in a Mini gel electrophoresis (BIOMETRA) using 75volt and 150 mA for 45 min after the gel was covered with TAE buffer (Appendix 8.2) using Standard Power Pack P25 (BIOMETRA). The cDNA bands were visualized using the BIODOC ANALAYZ gel documentation system (BIOMETRA).

2.5 Experimental infection of susceptible chicks with field isolates of IBD:

2.5.1 Objective of the experiment:

To study clinical signs, post mortem findings, morbidity and mortality of three different IBDV isolates.

2.5.2 Chicks:

All chicks used in experiment were Hisex chicks obtained from Ayaad Hatchery Khartoum north – Shambat as one day old chicks and reared in the experimental pens of Department of Microbiology, Faculty of Veterinary Medicine – University of Khartoum.
2.5.3 The experiment:

Three groups of non-vaccinated chicks A, B and C (35 chick in each group) were inoculated orally by 10% bursal homogenate (0.2ml/bird) at the age of 22 days. Three IBDV isolates were selected for experimental infection based on difference in epidemiology and clinical picture. Group A infected with Omdurman isolate, group B infected with Alkabashi isolate and group C infected with Butri isolate. Morbidity and mortality were recorded daily for 10 days. A fourth group of 20 chicks was left as uninfected control (group D). Bursal samples were taken from infected groups and tested by AGID against positive known hyper immune serum for IBDV.
CHAPTER III

RESULTS

3.1 Field visits:

A number of 30 outbreaks distributed in Khartoum state were investigated (Table 1). These farms follow different IBD vaccination programs as shown in Table 1.

3.2 Morbidity and mortality due to IBD:

The overall morbidity in the investigated outbreaks was estimated to be between 70–90% with a mean of 80% (Table 1), while the mortality varied between 26–76% with a mean of 51% depending on the area, age at infection and probably the challenge virus (Table 1).
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<th>No of vaccination doses</th>
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† Manufactured by INTERVET, Holland.
♣ Manufactured by Merial, France.
*Manufactured by Isovac, Belgium.
Table 2: Comparison between commonly used IBDV vaccines in the Sudan:

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<td>Intermediate</td>
<td>Hot</td>
<td>Intermediate plus</td>
</tr>
<tr>
<td>Mortality percentage</td>
<td>42 %</td>
<td>34 %</td>
<td>41 %</td>
</tr>
</tbody>
</table>

Note that 228E is not a moderate vaccine but an invasive or hot vaccine and it have the less percentage of mortality.

Figure (1). A diagram that shows the distribution of IBD outbreaks in different age groups that occurs in Khartoum state 2003 to 2004.
Figure (2) A photo that shows chicks infected with IBDV taken on day 3 after the onset of the disease. Note ruffled feather and depression of affected birds.

3.3 Clinical signs:
In all investigated flocks the disease was characterized by sudden onset of depression. The affected birds showed signs of dullness, ruffled feather, reluctant to move, watery diarrhea, recumbency with the beak on the earth, off food and pasted vent.

Figure (3). An affected bird with IBD showing dullness, ruffled feather, reluctant to move.
3.4 Post mortem findings:

Post mortem examination of the affected birds revealed dehydration of carcasses, haemorrhages in skeletal muscles (thigh & pectorals muscles) (Figure 4), in some cases there were haemorrhages in the junction of proventriculus and gizzard. Kidneys showed enlargement with distended tubules containing urates. The bursa of fabricius enlarged (2 – 3 times) (Figure 5) and become odematous, yellowish and some times hemorrhagic (figure 5).

Figure (4) Hemorrhages in skeletal muscles in IBD affected bird.
3.5 Histopathological findings:

Most of the pathological lesions occurred in the bursa of fabricius. Severe lymphoid necrosis and depletion occurred in both medulla and cortex of the follicles. Severe edema with heavy infiltration of heterophils and macrophages were also observed (Figure 6 and 7).
Figure (6): Histopathological section of bursa of fabricius of IBD affected bird showing different sizes of follicles and cracking of follicles with increase of interfollicular tissue (signs of degeneration). (H & E) x 10
Figure (7): Histopathological section of affected bursa of fabricius showing degeneration of lymphocytes (Lymphoid depletion) (H & E) x 40
3.6 Laboratory Diagnosis:

3.6.1 AGID:

IBDV was identified in the 10% bursal homogenates of 22 samples out of 30 by AGID. The samples gave precipitin lines with the known antisera against IBDV indicating the presence of IBDV antigen in the bursal homogenate. 22 samples out of 30 samples were clear positive by AGDT test.

3.6.2 Counterimmunoelectrophoresis (CIEP) test:

IBDV antigens were identified in bursal homogenates by using CIEP test. 18 samples out of 30 gave clear precipitin lines (Table 2).

3.6.2 Virus isolation:

3.6.2.1 Inoculation of embryonated eggs:

No lesions were produced by inoculation of CAM & yolk sac of chicken embryonated eggs with 10% bursal homogenate. A number of 3– 4 passages and no lesions were seen. Each passage was tested by AGID against known antisera. No precipitin lines were seen between the homogenized CAMs and the antisera.

3.6.2.2 Cell culture:

A number of 10 AGID positive samples were selected and inoculated onto chicken embryo fibroblast (CEF) cell culture. No lesions or any morphological changes were seen in CEF cell culture after inoculation even after four blind passages. Each passage was tested using AGID against known antisera. No precipitin lines were seen.
3.7 Reveres- transcription polymerase Chain Reaction (RT-PCR):

RT-PCR was conducted on RNA samples extracted from infected bursas collected from field outbreaks using RNA isolation kits (PUREGENE) and TRIzol method.

The RT-PCR was done with primer IBDV-F and IBDV-R. No amplification products were detected when control negative (RNA prepared from normal bursa and DDW) were used as templates while the control positive (D78 vaccine) gave positive results. A number of 14 out of 15 RNA samples extracted from infected bursas collected from thick birds gave positive result for the VP2 gene. The bands were detected in the ethidium bromide stained gel that correspond exactly to the expected cDNA band size of the control positive 479 bp. Figure (8).
Figure (8): Ethidium bromide stained Agarose gel (1%). RT-PCR was carried out on RNA samples extracted from bursa of infected birds using primers IBDV-F/IBDV-R lane M: 100bp ladder, lane 1 control positive (D78 vaccine), lane 2, lane 3, lane 4 bursas sample from field. Lane 5 control negative (bursa from uninfected bird) lane 6 control negative (DDW).
Table (3): Comparison between AGDT, CIEP, PCR and virus isolation in cell culture and embryonated eggs for diagnosis of IBD.

<table>
<thead>
<tr>
<th></th>
<th>AGDT</th>
<th>CIEP</th>
<th>PCR</th>
<th>Cell culture</th>
<th>Embryonated eggs</th>
</tr>
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<tbody>
<tr>
<td>No tested</td>
<td>30</td>
<td>30</td>
<td>15</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>No positive</td>
<td>25 (83.4 %)</td>
<td>20 (66.7 %)</td>
<td>14 (93.3 %)</td>
<td>0 (0 %)</td>
<td>0 (0 %)</td>
</tr>
<tr>
<td>No negative</td>
<td>5 (16.6 %)</td>
<td>10 (33.3 %)</td>
<td>1 (6.7 %)</td>
<td>15 (100 %)</td>
<td>15 (100 %)</td>
</tr>
</tbody>
</table>

3.8 Experimental infection of susceptible chicks with field isolates of IBDV:

3.8.1 Incubation period of IBDV:

The experimental incubation period of IBD in experimentally infected chicks was found to be 2-3 days. Experimentally only 6% of inoculated birds show signs of depression, dullness and ruffled feather after 36 hours PI.

Table 3 shows the morbidity & mortality rate of the 3 groups inoculated with different IBDV isolates.

Table 4: Results of experimental infection of chicks with IBDV local isolates.
<table>
<thead>
<tr>
<th>Day</th>
<th>PI</th>
<th>Morbidity</th>
<th>Group A</th>
<th>Group B</th>
<th>Group C</th>
<th>Control</th>
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<tr>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td></td>
<td>Morbidity</td>
<td>No signs</td>
<td>No signs</td>
<td>No signs</td>
<td>No signs</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mortality</td>
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<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Day 2</td>
<td></td>
<td>Morbidity</td>
<td>No signs</td>
<td>No signs</td>
<td>2 birds</td>
<td>No signs</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>show signs</td>
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<td></td>
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<td>No</td>
<td>No</td>
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<td>Day 3</td>
<td></td>
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<td>8</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mortality</td>
<td>No</td>
<td>No</td>
<td>2</td>
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</tr>
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</tr>
<tr>
<td></td>
<td></td>
<td>Mortality</td>
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<td>3</td>
<td>3</td>
<td>No</td>
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<td>Day 5</td>
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<td>3</td>
<td>1</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>No</td>
<td>1</td>
<td>No</td>
</tr>
<tr>
<td>Day 6</td>
<td></td>
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<td>No</td>
<td>No</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Mortality</td>
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<td>No</td>
</tr>
<tr>
<td>Day 7</td>
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<td>No</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Mortality</td>
<td>No</td>
<td>2</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Day 8</td>
<td></td>
<td>Morbidity</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Day 9</td>
<td></td>
<td>Morbidity</td>
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<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mortality</td>
<td>No</td>
<td>2</td>
<td>No</td>
<td>No</td>
</tr>
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</table>
CHAPTER IV
DISCUSSION

Infectious bursal disease or Gumboro is an acute highly contagious viral disease of young chickens which lead to immunosuppression and great economic losses (Mittal et al., 2005).

Immunosuppressive viral diseases are a great concern for poultry industry for several years. Indeed the reemergence of IBDV in variant or highly virulent forms have been the cause of significant losses (Paul et al., 2004). In addition the increase use of antibiotics to fight opportunistic (secondary) infection is a major concern to human health, if we consider the risk linked to the presence or residues in meat products, the release of the residues in the environments and increased antibiotic resistance (Paul et al., 2004).

The two main objectives of this study were to determine the epidemiology of recent IBD infection in chicks in Khartoum state and to introduce and compare RT-PCR with classical techniques for diagnosis. Field outbreaks of IBDV (n=30) that occurred between February 2003 and June 2004 in Khartoum state were studied and the clinical signs of IBD were similar to those observed after experimental infection of susceptible chicks. The signs were characterized by sudden onset of depression, dullness, ruffled feather, reluctance to move, watery diarrhea, recumbency with the beak on the earth, off food and pasted vent. Theses signs are similar to those described in the early outbreaks of IBD by Cosgrove, (1962) and also to that described by Parkhurst (1964). In the field, the mortality ranged between 26 – 76% with a mean of 51%. These mortality rates are closer the percentages that described by Chettle et al (1989), Nunoya et al
(1992) and Di Fabio et al (1999) caused by the vvIBDV. However, the mortality rates reported in this study are lower than that described by van den Berg et al (1991) who reported a mortality rate between 70-100%, but it is higher than mortality rates described by Rosenberger et al (1986). Moreover, the morbidity and mortality rates described in the present outbreaks are much higher than that reported during previous outbreaks of IBD in the Sudan which described by Shuaib et al (1982); Gaffer et al (1988); Khalafalla et al (1990). This mortality pattern point to increased severity of IBDV in the Sudan or they may support the assumption of introduction of the vvIBDV to the country. Research work that can compare old and recent isolates of IBDV is highly needed to prove this hypothesis.

In the investigated outbreaks it was noticeable that there was a high mortality rate despite vaccination against the disease (96.7% of flocks were vaccinated). This finding denotes to a vaccination failure which might be due to emergence of a vvIBDV that is antigenically different from the virus in the vaccines used (Snyder, 1990). The vvIBDV strains could establish infection in the presence of high level of maternal antibodies that were protective against classical strains as indicated by Chettle et al (1989) and cause up to 60-100% mortality (van den Berg et al, 1991). The vaccination failure may also be due to improper timing of vaccination or faulty application of vaccines. These points highlight the need for genetic comparison of field and vaccinal virus genomes by partial or complete sequencing and cross serological tests by VNT or ELISA.

It is worth mentioning that the mortality rate in birds vaccinated with the hot IBD vaccine (228E) was lower than that recorded in birds vaccinated by intermediate strains (Table 4). The Hot vaccines are less attenuated and better able to cope with the field
challenge by establishing strong ‘Immunity Cushing’ in the infected areas and these findings were similar to that were found by Mazariegos et al (1990) and Lukert & Saif (1991).

Histopathologically, the examined bursas showed infiltration by lymphocytes and necrosis of the plasma and the plast cells which resulted in destruction of all lymphoid tissue in the bursa leaving reticular structure of follicles. These results agree with that of Okoye (1983); Lang et al (1987); Lasher & Shane (1994) and Egbal (2001).

Experimental inoculation of susceptible 22-days old chicks with bursal homogenates collected from field outbreaks that caused 26%, 60% and 37% mortality rates resulted in 0%, 23% and 17% mortality rates, respectively. It is apparent from these results that virulence of the field virus has remarkably reduced during this single chick passage. Taking into consideration that birds infected in the field outbreaks were vaccinated against the disease while the experimental chicks were not, it seems difficult to explain what happened. However, there are some possible explanations, one being that the experimental chicks had a kind of transient exposure with a low virulent IBDV strain that resulted in some sort of immunity. Low virulent strains of IBDV were previously reported in the Sudan (Khalafalla et al., 1990) and subclinical IBD occurs when chickens are exposed to IBDV during the first two weeks post hatch (Mcllory, 1994). Sera collected before inoculation and tested by AGID test revealed no antibody titer. However, resistance to infection could be due to effective local immunity, which is quite different from the humoral immune response. Another explanation could be that the IBD infection in the field depends on a combination of environmental and biological factors that are not considered in the experiment infection. According to Chang and Hamilton (1982)
morbidity and mortality depend on the virulence of the challenged virus, the immune status and age of the infected birds and other factors affecting the pathogenicity of IBDV like aflatoxins infection. A third explanation could be that clinical IBD disease is a result of genetic reassortment of more than virus strains the coexist in the environment. IBDV virus is a bi-segmented virus and such genetic reassortment could occur in the field (Islam et al., 2001). Further research is needed to examine these possibilities.

The classical techniques that are commonly used in the diagnosis of IBD in Sudan are AGID and virus isolation and identification. The AGID test is highly specific for IBDV precipitins in that no false positive reaction occurs, but it is less sensitive and may give false negative as indicated by Walter (1981). The virus isolation is laborious, non-specific and time-consuming; besides, the recent very virulent strains of IBDV can not be grown in cell culture.

In the present study, classical techniques of AGID, CIEP and virus isolation in embryonated eggs and cell culture were used for the diagnosis of field cases. 22 samples out of 30 samples (73%) were clearly positive by AGID test and 18 samples out of 30 (60%) gave clear precipitin lines by using CIEP test. This result indicates that the AGID test is more sensitive than CIEP in detecting IBDV antigens.

On the other hand, no lesions were produced by inoculation of 15 samples onto the CAM and yolk sac of chicken embryonated eggs with 10% bursal homogenate of field samples. A number of 15 AGID positive samples were selected and inoculated onto monolayers of CEF cell culture. No lesions or any morphological changes were seen in CEF cell culture after inoculation even after four blind passages. On the other hand, no lesions were obtained when the processed bursal homogenates were inoculated onto the
CAMs of 11-13 day old embryonated eggs. These findings point to inability to grow Sudanese isolates of IBDV in embryonated eggs and cell culture and render this technique not suitable for routine diagnosis. These findings are not unexpected since Bumstead et al (1993) and Islam (2002) reported similar findings and argue that the vvIBDV do not grow in cell culture.

It is therefore, virus isolation and identification are is not recommended for the diagnosis of IBD as indicated in the present study

The classical techniques of AGID, CIEP were compared with the recent molecular biological technique of PCR in an attempt to determine the feasibility of using PCR for rapid and accurate diagnosis of the in the Sudan.

In the present study the RT-PCR (using bursa samples from field outbreaks) was used successfully to amplify the IBDV VP2 gene region with oligonucleotide primers selected from highly conserved region. The oligonucleotide primers used in this study successfully directed the synthesis of IBDV genome sequence, yielding amplified fragment of 479 bp confirming the results of Darja et al (2002).

A number of 14 out of 15 samples were positive by RT-PCR. The RT-PCR is found to be more sensitive since it detected 93.3% of tested samples in comparison to AGID and CIEF which detected 83.4% and 66.7% of tested samples, respectively.

Classical methods have disadvantages like lacking of the ability to detect low levels of IBDV in tissues. The RT-PCR is rapid, specific, and highly sensitive and there is no need to grow the virus even if it is in low levels and the loss of virus infectivity does not affect the RT-PCR as indicated by Mittal et al (2005).
The use of PCR for the diagnosis of IBD was established by Davis & Boyle (1990), Jackwood (1990) and Wu et al (1992). Currently, the RT-PCR is the molecular method more frequently used in the diagnosis of IBD.

However, this test is relatively expensive since it requires costly reagents like RNA extraction kit, RT-PCR kit, agarose and buffer for electrophoresis and gel documentation system to visualize the RT-PCR product and record the results. This high cost is not affordable to most small poultry producers in the Sudan at present. To the well organized large poultry farms that seek reliable and quick tests, the PCR is an option, because of the intensive production system and the high inputs invested. Efforts should be continued to reduce the cost of performing the PCR in the Sudan through standardization of the test for each disease and the use of locally prepared reagents.

The PCR technique is currently used for the diagnosis of various viral disease in the Sudan, the present study established the diagnosis of IBD by PCR technique in the Sudan.

CONCLUSIONS
1. The vvIBDV is responsible for the recent outbreaks of the disease in the Sudan that resulted in a mean high mortality rate of 51% even in vaccinated flocks.

2. Diagnosis of IBD by classical techniques is laborious and time consuming, and the introduction of PCR in the Sudan is of a great value in diagnosis of diseases, because it is a rapid, reliable, highly specific and sensitive technique.
RECOMMENDATIONS

1. Further research work should be carried out to molecularly characterized IBDV strains through sequencing in comparison to the vaccines in use in the country.

2. Local production of IBD vaccine through attenuation of a local isolate of the virus in cell culture or inactivation

3. Improving and optimization of the RT-PCR and consider it as a routine technique in diagnosis of IBD in the Sudan.

4. Revision of IBD vaccination programs in the Sudan taking into consideration results of this study.
REFERENCES


**Faragher, J. T., Allan, W. H. and Wyeth, P. J. (1974).** The immunosuppressive effect of infectious bursal agent on chickens vaccinated against Newcastle disease. The Veterinary Record 95, 385-388.


infectious bursal disease (IBD) and Chicken infectious anaemia (CIA), 197-205. Rauschholzhansen, Germany, June 1994.


APPENDIX

1. Preparation of Reagents and Solutions:

1.1 Normal Saline (NS):

Stock solution of 0.85% (W/V) NaCl was prepared in DDW and autoclaved at 121 °C for 20 minutes.

1.2 Phosphate buffered saline (PBS):

Solution A:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>16gm</td>
</tr>
<tr>
<td>KCl</td>
<td>0.4gm</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$</td>
<td>2.3gm</td>
</tr>
<tr>
<td>KHPo$_4$</td>
<td>0.4gm</td>
</tr>
</tbody>
</table>

Complete to 1500ml by DDW

Solution B:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrous MgCl$_2$6H$_2$O</td>
<td>0.42gm</td>
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</table>

Or

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anhydrous MgCl$_2$</td>
<td>0.2gm</td>
</tr>
</tbody>
</table>

Complete to 200ml by DDW
**Solution C:**

CaCl anhydrous 0.2gm

Or

CaCl2H2O hydrous 0.264gm

Complete to 200ml.

Each solution was autoclaved separately and solution A and B were added to C, then was completed to 2 liters by adding sterile DDW.

**1.3 Preparation of phosphate diluents (PD):**

Solution A of PBS was completed to 2 liters with DDW, it was then autoclaved and cooled before antibiotic were added.

**1.4 Preparation of Stock Trypsin (2.5%):**

2.5 grams of trypsin (Gibcol tdUK1:250UspGrade) were dissolve in 100ml of PD, filtered through What Man filter and stored at -20 °C.

**1.5 Preparation of Stock Versin (5%):**

5 gram of versin powder was dissolved in 100ml PD, autoclaved at 121°C for 15 minutes.
1.6 Preparation Trypsin Versin solution:

6ml of trypsin (2.5%) was added to 4ml of version (5%), completed to 100ml with phosphate diluents, with addition of few drops of phenol red were added pH adjusted by NaOH.

1.7 Preparation of 100 ml buffer AGID test:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>8 gm</td>
</tr>
<tr>
<td>Phenol</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>DDW complete to</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

2. Preparation of Media Additives:

2.1. Preparation of Tryptose phosphate broth (29.5%):

Twenty-nine and half gm of TPB powder were dissolved in 100ml of DDW, autoclaved at 121°C for 10 minutes and stored at 4°C.

2.2. Preparation of Lactalbumin hydrolysate (5%):

Five grams of lactalbumin powder were dissolved in 100ml of DDW, sterilized by autoclaving at 121°C for 10 minutes stored at 4°C.

2.3. Preparation of Yeast extracts solution (1%):
One gram of yeast extract powder was dissolved in 100ml of DDW, autoclaved at 121°C for 10 minutes and stored at 4°

2.4. Preparation of Sodium bicarbonate (NaHCo3) (7.5%):

Seven and half grams of NaHCo3 were dissolved 100ml of DDW, by autoclaving at 121°C for 10 minutes.

2.5 Bovine serum:

Calves were bled from the jugular vein. The whole blood was left overnight at room temperature serum was centrifuged at 2000 rpm for 10 minutes, and then filtered through Seitz filter under negative pressure, tested for sterility using thioglycolate media and kept at –20°C.

3. Preparation of antibiotic solutions:

One gram of streptomycin powder and the contents of 2 vials of penicillin (1000000 IU/ vial) were dissolved in 10ml of sterile DDW so that 1ml of the prepared solution contained 100mg streptomycin and 2000000 IU penicillin. The solution was kept at -20 °C.

4. Mycostatin:

One vial of Mycostatin (50000ug) was dissolved in ten ml of sterile DDW so that 1ml of the solution contained 5000ug.

5. Preparation and sterilization of media and solutions:
To prepare the five fold stock solution, 125.19gm of Glasgow Modified Eagles Medium (GMEM) powder were dissolved in two litres of water. The solution was filtered through Millipore filter (0.22u) under positive pressure, tested for sterility using thioglycolate medium and stored at – 20°C.

5.1 Growth and Maintenance media:

- GMEM x 5 concentration: 200ml
- 0.5% lactalbumin hydrolysate: 25ml
- 1% yeast extract: 25ml
- Tryptose phosphate broth: 25ml
- 7.5% NaHCO₃: 8ml
- Penicillin / streptomycin: 1ml
- Fungizone: 1ml

DDW was added to complete to one litre. For preparation of growth media 100ml of calf were added, 50 ml for maintenance.

6. Preparation of Thioglycolate medium:

Twenty nine and half gram of thioglycolate medium was dissolved in 1000ml of DDW. The solution was put in steamer to ensure complete dissolving and dispensed in bijoux bottles. The medium was sterilization by autoclaving at 121 C° for 10 minutes.

7. Preparation of Buffers:

7.1 Glycerol (50%):
Glycerol  50ml
PBS  50ml

7.2 Formalin (10\%):

Formalin  200ml
NaH₂PO₄  4gm
K₂HPO₄  6gm

Complete to one litre with DDW.

8. Preparation of materials for gel electrophoresis:

8.1 Agarose 1\%

1gm of Agarose (SIGMA) was dissolved in 100ml TAE buffer heated in a microwave for 45 seconds, left to cool and poured onto electrophoresis chamber.

8.2 TAE Buffer:

40m M Tris – HCl (pH 8.5)  40ml
20 m M Na-acetate  20 ml
EDTA Powder (292.25 MW)  5845gm

Complete to one litre.

8.3 Loading dye:
Bromophenol Blue (11%)
Glycerol 40ul
DDW 50ul

8.4 100 bp Ladder (INVITROGEN):
Ladder (1.0 ug/ul) 20ml
Blue dye 80ml

8.5 Ethidium bromide:
Stock solution (PROMEGA, Madison) 10mg/ml
And protected from light.

Preparation of 500ml buffer for IBDV AGID:
NaCl 40g
Phenol 2.5ml
DDW make to 500ml