A SURVEY FOR CHICKEN INFECTIOUS ANAEMIA AND INFECTIOUS BURSAL DISEASE IN ZARIA AND ENVIRONS, KADUNA STATE, NIGERIA

BY

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BY

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DECLARATION

I declare that the work in this Dissertation entitled, "A survey for chicken infectious anaemia and infectious bursal disease in Zaria and environs, Kaduna State, Nigeria" has been carried out by me in the Department of Veterinary Medicine, Ahmadu Bello University Zaria. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this Dissertation was previously presented for another degree or diploma at this or any other institution.

Anthony Achile OKPANACHI	____________________	____________
Student	Signature	Date

CERTIFICATION

This Dissertation entitled, "A SURVEY FOR CHICKEN INFECTIOUS ANAEMIA AND INFECTIOUS BURSAL DISEASE IN ZARIA AND ENVIRONS, KADUNA STATE, NIGERIA" by Anthony Achile OKPANACHI, meets the regulation governing the award of the degree of Master of Science of Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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ABSTRACT

Chicken infectious anaemia (CIA) is a contagious viral disease of primarily young chickens characterized by severe anaemia, high mortality, co-infection with infectious bursal disease virus or other pathogens, severe immunosupression, susceptibility to other infectious agents and economic losses. Although, CIA virus (CIAV) and antibody have been detected in south west Nigeria, information on CIA in Zaria and its environs is lacking. The study was carried out to detect the presence of CIA and infectious bursal disease (IBD) viruses in chickens, to determine the seroprevalence of CIA and IBD in Zaria, and to detect possible co-infection of CIAV and IBD virus (IBDV) in chickens in Zaria and environs, Nigeria. A total of 351 chicken sera (from 93 breeders, 109 layers and 149 broilers) were collected from flocks in Zaria and tested for CIA antibodies, 346 of these sera were tested for IBD antibodies. A total of 69 bursae and 63 thymuses were collected and tested for CIA and IBD virus. Enzyme linked immunosorbent assay was used to detect antibodies against CIA. Agar gel immunodiffusion test was used to detect IBD antibodies in sera and IBDV antigens in thymus and bursae. Polymerase chain reaction was used to test the bursae and thymus for CIAV antigens. The seroprevalence of CIA was 58.97% (207/351), seroprevalence of IBD was 33.2% (115/346), prevalence of IBDV viruses was 23.2% (16/69), while the prevalence of CIAV antigens in the thymuses was 3.17% (2/63) and 1.45% in the bursa (1/69). Both IBD and CIA antibodies were detected in 38.3% of the bursae sampled. Only 3.4% (1/29) of flock were infected with both CIAV and IBDV. It was 7.29 times significantly ($p < 0.05$) more likely to detect antibodies to CIA in adult ($\geq$ 19 weeks) than in young ($\leq$ 6 weeks) chickens. In conclusion, CIAV (3.17%) and IBDV (23.2%) were detected in chickens in Zaria and environs, Nigeria and seroprevalence of

CIA and IBD in chickens in Zaria were 58.97% and 33.2% respectively. It is recommended that, for subsequent research sample size of bursae and thymus should be increased in order to establish more accurately the probability of co-infection of CIAV and IBDV in Zaria and environs. Further research should be carried out to characterize the CIAV detected in Zaria. Improved biosecurity practices are recommended on poultry farms in order to prevent infection by CIA and IBDV. Breeders should be vaccinated against CIA to prevent infection which could lead to vertical transmission of CIAV and to confer maternal antibodies to their offspring.

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LIST OF ABBREVIATIONS AND SYMBOLS

° F	Degree Fahrenheit
°C	Degree centrigrade
µL	Microliter
ab	Antibody
AGID	Agar gel immunodiffusion
B- cell	B lymphocyte
bp	Base pairs
CAV	Chicken anaemia virus
CIA	Chicken infectious anaemia
CIAV	Chicken infectious anaemia virus
CL	Critical level
CL-1	Chicken anaemia agent (CAA) isolated by Lamichhane *et al*.
df	Degree of freedom
DNA	Deoxyribonucleic acid
E	East
EINIB	Eosinophilic intranuclear inclusion bodies
ELISA	Enzyme-linked immunosorbent assay
FFPE	Formalin fixed paraffin- embedded sections
IBD	Infectious bursal disease
K Da	Kilo Dalton
kb	Kilobase
L	Ladder
ml	Mililiter
Mm	Milimolar

N	North
Nm	Nanometer
OR	Odds ratio
ORFs	Overlapping open reading frames
P- value	Probability
PCR	Polymerase chain reaction
pH	Power of hydrogen/ Power of concentration of the hydrogen ion
rpm	Revolutions per minute
SPF	Specific- pathogen free
T-cell	T lymphocyte
$TCID_{50}$	Median tissue culture infective dose
UK	United Kingdom
VP1	Viral protein one
VP2	Viral protein two
VP3	Viral protein three
Z	Z score
α	Alpha

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Chicken infectious anaemia (CIA) is a contagious disease of primarily young chickens characterized by severe anaemia, generalized lymphoid atrophy, increased mortality in young chicks with accompanying severe immunosuppression that enhances susceptibility to other infectious agents, reduced response to vaccines and serious economic losses (Hagood *et al*., 2000). Diagnosis of clinical CIA in broilers is easy due to the age of its onset and clinical signs (Fussel, 1998).

The causative agent of CIA is the chicken infectious anaemia virus (CIAV) and is found to be ubiquitous in all major chicken producing countries of the world with evidence from serology and virus isolation (Bulow, 1991; McNulty, 1991; Zhou *et al.*, 1997). The virus was first described in 1979 by Yuasa *et al*. (1979) in commercially produced chickens. The virus has been isolated from both layer and broiler chickens (Bulow and Schat, 1997).

Chicken infectious anaemia virus is economically significant because the clinical disease is vertically transmitted and because of its ability to cause immunosuppression by itself or in combination with other pathogens (Rosenberger and Cloud, 1998). Examples of such infectious agents are infectious bursal disease virus (IBDV) and gallid herpesvirus 2

(Mahzounieh *et al*., 2005). A highly significant association has been demonstrated between CIAV infection and other diseases (Hagood *et al*., 2000).

Chicken infectious anaemia virus has been found in chickens in Japan, China, many European countries, the United States, South Africa, Australia, New Zealand and South Western Nigeria (Saif *et al*., 2003; Owoade *et al*., 2004).

Various commercially available enzyme-linked immunosorbent assay (ELISA) techniques for the detection and measurement of CIA antibodies in chicken sera have been developed. These tests are routinely used to screen breeder flocks for CIAV in countries where vaccines are available (Engstrom, 1999).

1.2 Statement of the Research Problem

Infection with CIAV constitutes a serious economic threat, due to immunosuppression. This threat occurs especially to the broiler industry and the producers of specific-pathogen free (SPF) eggs where vertical transmission of the virus to offspring chicks usually occurs (McIlroy e*t al*., 1992).

In Nigeria, CIAV has been suspected to be involved in outbreaks of disease in chickens characterized by clinical signs and pathology, such as depression, ruffled feathers, muscular haemorrhages and bursal atrophy, which are associated with infectious bursal disease (IBD) (Oluwayelu *et al*., 2005).

Also, the suppressive effects of CIAV on immune function have been associated with reduced vaccinal immune responses to Marek's disease, Newcastle disease and infectious laryngotracheitis vaccines (Otaki *et al*., 1988a; Cloud *et al*.,1992a; DeBoer *et al*., 1994). Furthermore, CIAV causes an enhancement in pathogenicity of a wide range of co-infecting agents, like Marek's disease virus (Bulow *et al*., 1983; Otaki *et al*., 1987, 1988b, 1989; Jeurissen and DeBoer, 1993), reovirus (Engstrom *et al*., 1988; McNeilly *et al*., 1995), IBDV (Yuasa *et al*., 1980a; Otaki *et al*., 1989; Rosenberger and Cloud, 1989a; Cloud *et al*., 1992b), Newcastle disease virus (De Boer *et al*., 1994), *Staphylococcus aureus* and *Cryptosporidium* (Dobos-Kovacs *et al*., 1994; Hornok *et al*., 1998; Mc Namee *et al*., 1999).

1.3 Justification of the Research

The chicken infectious anaemia virus has been isolated in many countries, since the first description of the disease and isolation of the virus in Japan (McNulty *et al.*, 1989). In Nigeria, previous serologic surveys have detected the presence of CIAV-specific antibodies in indigenous and commercial chickens (Owoade *et al*., 2004; Emikpe *et al*., 2005).

Also, a report of mixed infection of both CIA and IBD in south western Nigeria (Owoade *et al*., 2010) makes it imperative to investigate this phenomenon in Zaria and environs. Chicken infectious anaemia is rarely diagnosed or suspected in veterinary clinics in Nigeria due to the similarity of CIA to IBD in terms of pathological findings which probably makes many clinicians to diagnose such cases at post mortem only as IBD. This study will be necessary to ascertain the presence of CIA in chickens in Zaria and environs, north western Nigeria.

Since aplastic anaemia, immunosuppression and complications of secondary viral, bacterial, or fungal infections are common findings in CIA (Schat, 2003) it is necessary to carry out research on CIA to establish its current status in Zaria and its environs, Nigeria.

To the best of our knowledge, there is no published paper on the presence of CIA in Zaria and its environs, Kaduna State of north western Nigeria.

The present survey for CIA and IBD using serological methods and polymerase chain reaction will provide information about the prevalence of these diseases in Zaria and environs, Kaduna State of Nigeria.

1.4 Aim of the Study

The aim of this study was to determine the relationship between CIA and infectious IBD in chickens in Zaria and their prevalences.

1.5 Objectives of the Study

The objectives of the study were to:

- detect the presence of CIA and IBD virus in chickens in Zaria and environs, Nigeria.
- determine if there is co-infection of CIA and IBD virus in chickens in Zaria, Nigeria.
- determine the seroprevalence of CIA and IBD in Zaria and environs, Nigeria.

1.6 Research Questions

Are CIA virus and IBD virus present in chickens in Zaria?

Are chickens in Zaria co-infected with chicken infectious anaemia virus and infectious bursal disease virus?

What is the seroprevalence of CIA and IBD in chickens in Zaria?

CHAPTER TWO

LITERATURE REVIEW

2.1 Chicken Infectious Anaemia

2.1.1 Aetiologic agent of chicken infectious anaemia

Chicken infectious anaemia virus is the aetiologic agent of CIA (Eltahir *et al.*, 2011a). Chicken infectious anaemia virus is a Circovirus. Circoviruses are small, nonenveloped icosahedral animal viruses that are characterized by negative-sense circular single stranded DNA genomes. Their genomes are the smallest possessed by animal viruses. Chicken infectious anaemia virus is a member of the family *Circoviridae* (Todd *et al.,*1990; Eltahir *et al*., 2011b). In the family *Circoviridae*, different genera are distinguished by differences in genomic organization with the CIAV in the genus, Gyrovirus. The CIAV has protruding pentagonal trumpet- shaped units (Crowther *et al*., 2003). When the virus was examined by negative contrast electron microscopy the presence of spherical or hexagonal virus-like particles ranging from 18 to 22 nm in diameter was revealed (McNulty, 1991). The CIAV genome single-stranded circular DNA, size was estimated by electron microscopy to be 2.170 ± 0.043 (SE) (Todd *et al*., 1990) and 2.174 kb (Gelderblom *et al*., 1989) by electron microscopy and 2.3 kb electrophoresis in alkaline agarose gels (Todd *et al*., 1990).

The CIAV genome consists of 2.3 kb, with three partially overlapping open reading frames (ORFs), VP1, the major viral structural protein (51.6 k Da); VP2, a scaffolding protein (249 k Da); and VP3, a non-structural protein named apoptin (13.6 k Da) for its ability to induce apoptosis; VP1 and VP2 are the main targets of neutralizing antibodies (Noteborn *et al*., 1992, Eltahir *et al.*, 2011b). Only one serotype of the CIA virus has been isolated worldwide (McNulty, 1991; Ducatez *et al*., 2006).

The CIAV was first described in Japan in 1979. The CIAV is present in both layer and broiler breeds worldwide. The CIAV is very small and resistant to chemical and physical treatment; it can resist a pH of 3.0 and heat at 80 °C for 30 minutes. These factors make the virus difficult to eliminate on a poultry farm once the premises become contaminated (Butcher, 1994). The only host the virus infect is the chicken. The CIAV can be tramsmitted both horizontally, from infected to susceptible birds, and vertically, through the eggs of seronegative, infected breeders to chicks. The virus can also be transmitted mechanically from farm to farm by people, equipment and vehicles (Butcher, 1994).

Chicken infectious anaemia virus putative intergenotypic recombinants have been reported to occur, previously (Eltahir *et al*., 2011b). This fact is based on the previous classification of CIAV sequences into three genotypes, but it is unknown whether intersubtype recombination occurs between four recently reported CIAV genotypes and five subtypes of genome sequences. However, Eltahir *et al*. (2011b) using phylogenetic analysis with computational recombination detection algorithms to investigate CIAV genomes found statistically significant evidence of intersubtype recombination in the parent-like and two putative CIAV recombinant sequences. The recombinant event was shown to occur between CIAV subgroup A1 and A2 sequences in the phylogenetic trees. They concluded that intersubtype recombination in CIAV genome sequences played a role in generating genetic diversity within the natural population of a CIAV.

2.1.2 Properties of chicken infectious anaemia virus

Characterisation of CIAV showed that it passed through membrane filters of 25 nm pore size, resisted exposure to pH of 3, ether or chloroform. The virus is not inactivated by heating at 70° C for 1 hour or 80 °C for 5 minutes (Yuasa *et al.*, 1979) but the infectivity of CIAV was destroyed by hypochlorite.

Chicken infectious anaemia virus does not grow in cultured monolayer cells derived from a variety of chicken and chicken embryo tissues, nor in a variety of commonly used mammalian cells (Yuasa *et al*., 1979; Yuasa, 1983; Rosenberger and Cloud, 1989b). It does grow in some lympoblastoid cell lines established from Marek's disease and lymphoid leukosis lymphomas (Yuasa, 1983). The lympoblastoid cell lines established from Marek's disease and lymphoid leukosis lymphomas (MDCC-MSB1) cells are the most commonly used and their origin is from a Marek's disease T- cell splenic lymphoma. The MDCC-MSB1 cells grow in suspension culture and require subculturing every 2 to 3 days. Cultures infected with chicken infectious anaemia virus exhibit a cytopathic effect that is characterized by appearance of enlarged, swollen cells, cell destruction, alkaline medium and inability to subculture. Chicken infectious anaemia virus can be propagated in chickens and chicken embryos and the disease tend to be more severe following *in-ovo* inoculation than in parenterally inoculated 1- day-old chicks (McNulty, 1991). .

2.1.3 Epidemiology of chicken infectious anaemia virus

The CIAV has been isolated from chickens in Asia (Japan) (Yuasa *et al.,* 1979), Europe (West Germany) (Bulow *et al*., 1983; Vielitz and Landgraf, 1988), Sweden (Engstrom *et al*., 1988), the UK (McNulty *et al*., 1990a), North America (the USA) (Goodwin *et al.*, 1989), South America (Brentano *et al*., 1991), Australia (Firth and Imai, 1990), South Africa (Wicht and Maharaj, 1993). Chicken infectious anaemia can be concluded to be present in all continents (McNulty, 1991). Owoade *et al*. (2004) reported the presence of CIAV antibodies in Nigeria.

Serological evidence shows that CIAV infections are widespread and there is high prevalence of antibody to CIAV in older birds, both in egg-laying and meat-type breeds (Bulow, 1988; McNulty *et al*., 1988, 1989; Rosenberger and Cloud, 1989a and b; Jorgensen, 1990; Lucio *et al*., 1990; Goodwin *et al*., 1990; Imai *et al*., 1993; Toro *et al*., 1994; Yuasa *et al*., 1985; Dren *et al*., 1996; Zhou *et al*., 1996). Todd *et al*. (2001) reported a low incidence of clinical disease and this is confirmed by the findings that the clinical disease is rare because of the widespread practice of vaccinating breeders, but the sub-clinical form of the disease is ubiquitous (Sommer and Cardona, 2003). However, outbreaks of anaemia dermatitis syndrome, the clinical condition caused by CIAV are comparatively rare (Todd *et al*., 2001).

The chicken is the only natural host of the virus because antibody to the virus could not be found in turkeys and ducks in a small survey of their sera (McNulty *et al.*, 1988). Farkas *et al.* (1998) were unable to demonstrate antibodies in sera from pigeons, ducks and crows from Japan.

The majority of chicks in many flocks have maternally derived antibodies to CIAV (McNulty, 1991). Maternal derived antibodies to CIAV usually disappear by about 3 weeks of age. At 8 to 12 weeks of age, most breeder flocks seroconvert (McNulty *et al*., 1988).

Progeny chicks infected vertically appear normal at hatching but show increased mortality and develop typical disease from 10 to 14 days of age (Chettle *et al*., 1989; Todd *et al*., 2001). A low percentage of breeder flocks come into lay without having been exposed to CIAV which explains why outbreaks of clinical disease due to CIAV are rare. Chicks develop an age resistance to experimentally induced disease due to CIAV. Age resistance is complete by the age of 2 weeks, but older birds are still susceptible to infection (McNulty *et al*., 1989; McNulty, 1991).

Co-infection with immunosuppressive viruses like Marek's disease virus, IBDV and reticuloendotheliosis virus act synergistically with CIAV in dual infections, thus increasing the severity of the disease which overcomes the protective effect of age and maternal antibody (Yuasa *et al*., 1980b; Otaki *et al*., 1987; 1988a and b, 1989; Rosenberger and Cloud, 1989b; McNulty, 1991).

2.1.4 Clinical signs of naturally occurring chicken infectious anaemia

Outbreaks of CIA mostly occur in broilers (Chettle *et al*., 1989). The first signs of disease usually occur towards the end of the second week of life. Affected birds become anorexic and depressed, with pallor of the comb and wattles, and ruffled feathers, and an increased daily mortality has been noted to follow these signs. The disease is reported to be acute with peak mortality that occurs within five to six days of onset of disease. Signs and mortality often declines to normal levels after a further five to six days (Engstrom and Luthman, 1984; Yuasa *et al*., 1987).

2.1.5 Pathological features of chicken infectious anaemia

The presence of other infectious agents in the naturally occurring outbreaks of CIA disease makes the lesions more varied and more severe when compared with the experimentally induced disease (Todd *et al*., 2001).

Lesions caused by CIAV include pale bone marrow, thymic, splenic and bursal atrophy. Birds often have characteristic skin lesions, which appear to be due to development of subcutaneous oedema and skin haemorrhages that often extend to the underlying muscles (Todd *et al*., 2001). The skin turns blue and breaks, releasing a sero-sanguinous fluid. Experimentally infected chicks do not show these lesions, and the lesions are probably due to interaction of CIAV with other agents like reovirus (Engstrom *et al*., 1988). The lesions mentioned above are susceptible to secondary bacterial infection that leads to gangrenous dermatitis (Vielitz and Landgraf, 1988). Fungal pneumonia or colibacillosis may affect birds in the infected flock (Goryo *et al*., 1987). Names such as blue wing disease, anaemia dermatitis syndrome, infectious anaemia and haemorrhagic syndrome were given to the disease due to the nature of the presenting signs and lesions of the field disease.

2.1.6 Microscopic lesions due to chicken infectious anaemia virus infection

Yuasa *et al*. (1979) observed the following lesions due to CIAV infection: liver had dilated sinusoids which contained serous exudates with swollen endothelial cells. Fatty degeneration and hyaline necrosis lacking infiltrating cells were observed around the central vein in chicks that died due to CIAV infection. Inclusion bodies were not recognized in hepatic cells. In bone marrow, replacement of all the haemopoietic tissue by adipose tissue in CIAV infected chicks. The thymus in all infected chicks showed diminished lymphocytes in the lobules due to destruction and were replaced by reticular cells. Taniguichi *et al*. (1982) observed hypoplasia or aplasia of haematopoietic cells in the bone marrow and severe depletion of lymphocytes in the lymphoid organs (thymus, bursa of Fabricius, spleen and caecal tonsils) during their experimental studies on CIA.

Goryo *et al.* (1987b) noticed severe aplasia with depletion of the erythrocytic and granulocytic series of the bone marrow cells from the intravascular and extravascular spaces during an outbreak of anaemia caused by CIAV in young chicks. Hyperplastic foci consisting of haemopoietic cells were scattered within the aplastic areas. The lymphoid organs like thymus, bursa of Fabricius and spleen also revealed lymphocytic depletion with reticular cell hyperplasia. Reticular cells of the bursa of Fabricius in chicks had amphophilic or eosinophilic intranuclear inclusion bodies (EINIB). The hepatic sinusoids were dilated frequently with serous fluid, fibrinoid thrombi and swollen endothelial cells. There were aggregations of lymphocytes in the interlobular connective tissue of some birds.

Otaki *et al*. (1988a) reported depletion of lymphocytes in the thymus and hyperplasia of immature granulocytic series or hypoplasia of haematopoietic cells in the bone marrow during their study on field infection of CIAV in chicks. Chettle *et al*. (1989) observed lesions of CIA in the spleen, bursa of Fabricius and thymus during an outbreak of disease in broiler chickens in England. The spleen showed a loss of lymphocytes in the germinal centres. The bursa of Fabricius also showed loss of lymphocytes from the lymphoid follicles and shrinkage of bursal plicae with cystic changes in the epithelium covering the plicae. The thymus revealed marked atrophy with loss of most of the lymphocytes from the cortical parenchyma. The livers showed liquefactive necrosis.

Goodwin *et al*. (1989) observed septic necrotizing dermatitis, hepatitis, cellulitis, myositis, nonfibrinopurulent necrotizing bursitis, thymic and spleenic lymphoid hypoplasia or atrophy. In some section of the thymus, iron-laden macrophages were present. Nuclear or cytoplasmic inclusion bodies or foreign materials were not seen. Goryo *et al*. (1989a, b) during their experimental study of CIAV in chicks reported hypoplasia or aplasia of the bone marrow, depletion of lymphocytes in the lymphoid organs and intranuclear inclusion bodies in the haematopoietic precursor cells, thymocytes and reticular cells of the thymus.

Brentano *et al*. (1991) studied microscopic lesions in the thymus characterized by extensive depletion of lymphocytes from the cortex with hyperplasia of the reticular cells, resulting in the complete loss of the thymus architecture. The changes in the bone marrow consisted of severe depletion of erythrocytic and granulocytic series of cells and proliferation of connective tissue. The bursa of Fabricius revealed atrophy of bursal follicles with decreased number of lymphocytes and occasional infiltration of heterophils and mild proliferation of connective tissue. Lamichhane *et al*. (1991) observed histological lesions in the thymus of CL-1 CIAV infected chicks. The thymus showed fibrovascular stroma with sparse population of small dark thymocytes without distinct cortical-medullary interface. Bursal follicles varied in size with depletion of lymphocytes. In the bone marrow lesions were characterized by marked depletion of the erythropoietic and granulopoietic compartments. Almost all haematopoietic tissue was replaced by lipocytes. Spleen rarely contained lymphoid follicles and showed relatively few lymphocytes scattered throughout the sections. The liver lesions consisted primarily of extravasated erythrocytes, fewer necrotic cells and micro-colonies of bacteria.

Toro *et al*. (1997) observed the following microscopic lesions in CIAV infected chickens: thymic cortical necrosis, medullary necrosis, lymphocyte depletion, eosinophilic intranuclear inclusion bodies (EINIB) and apoptosis. In the bursa of Fabricius mild necrosis, EINIB and lymphoid depletion were observed in most of the birds. Moderate to severe lymphoid depletion was observed in the spleen. Focal necrosis and apoptosis were also detected in the spleen. Liver showed focal necrotic lesions throughout the experimental period. The presence of EINIB and vacuolar degeneration of hepatoctye was also found in the liver.

Ledesma *et al*. (2001) observed intranuclear inclusion bodies in the precursor cells of the bone marrow in field cases of chicken infectious anaemia. In experimentally induced CIA in chicks they observed cortical atrophy in the thymus together with evidence of tissue regeneration in the bone marrow. Spackman *et al*. (2002) observed thymic lymphocyte depletion and bone marrow atrophy in experimentally induced CIA by Del- Ros and CIAV-7 strains.

2.1.7 Pathogenesis of chicken infectious anaemia

Following intramuscular inoculation of l-day-old chicks, the main sites of replication of CIAV are the hemocytoblasts in the bone marrow and precursor lymphocytes in the thymus. This stems mainly from immunocytochemical, ultrastructural (Goryo *et al*., 1989a, b; Jeurissen *et al*., 1992a, b), and virological (Yuasa *et al*., 1983b) examinations of birds infected with the Cux-1 or TK 5803 isolates (Hoop and Reece, 1991; Jeurissen *et al*., 1992a; Smyth *et al*., 1993). It seems likely that the anaemia results from a direct cytolytic effect of the chicken infectious anaemia virus on the erythroblast precursor cells in the bone marrow (Adair, 2000). The haemorrhages that are seen in some chicks may be due, at least in part, to destruction of thrombocytes resulting in impaired clotting of the blood, but it has been suggested that endothelial lesions and impaired liver function may be more important (Bulow and Schat, 1997). Chicken infectious anaemia virus in the thymus targets precursor T cells during development in the cortex (Jeurissen *et al*., 1989; Cloud *et al*., 1992a; Adair *et al*., 1993; Hu *et al*., 1993a; Adair, 2000). Apoptosis may be the cause of death of cortical lymphocytes, triggered by the VP3 protein (Jeurissen *et al*., 1992a; Noteborn *et al*., 1994, 1998). Smyth *et al*. (1993) also found infected T-cells in the spleen and other lymphoid organs, although the frequency of infection appeared to be less than in the thymic cortex. Experimental findings in which infected cells were detected at the same time in the thymus as they appeared in extrathymic sites, suggesting simultaneous and independent infection of precursor and mature T-cell populations in different organs (Smyth *et al*., 1993). There is no evidence that CIAV is capable of infecting B cells *in-vivo*, although infection of a B-cell line *in-vitro* has been described but using electron microscopy and biochemical methods, it was demonstrated that CIAV induces apoptosis of cortical thymocytes after *in-vivo* infection and of lymphoblastoid cell lines after *in-vitro* infection (Yuasa *et al.*,1983a; Jeurissen *et al*., 1992a).

Lack of susceptibility of the B-cell population to infection by CIAV is crucial for the survival and ultimate recovery of the infected birds. The B-cell response to CIAV infection is initiated as the bursa of Fabricius reaches full immunocompetence in the young birds.

The appearance of CIAV antibodies in the serum coincided with the disappearance of the virus from the blood, and from many organs and tissues, although infectivity appeared to persist in several tissues even in the presence of circulating antibodies (Yuasa *et al*., 1983b). The persistence of CIAV following experimental infection of birds which were previously bursectomized as embryos, and the more rapid clearance of virus from older birds (i.e. those infected with CIAV at 28 or 42 days old) has also suggested a primary role for antibody in the recovery process (Yuasa *et al*., 1983b; Hu *et al*., 1993b). Reduction of CIAV population leads to recovery of T- lymphocytes and other affected cells (Todd *et al*., 2001).

2.1.8 Immunosuppression caused by chicken infectioius anaemia

The secondary bacterial infections and fungal infections associated with chicken infectious anaemia indicates its immunosuppressive nature (McNulty, 1991; Bulow and Schat, 1997). There is evidence for the impaired immune function associated with CIAV infection (Todd *et al*., 2001). Evidence showed that lymphocyte functions, including response to mitogens and production of cytokines are substantially reduced for up to 3 to 4 weeks following CIAV infection of 1 day-old or 3-week-old SPF birds (Adair, 1996; 2000). The function of macrophages were seen to be impaired by infection with CIAV by both *in-vivo* and *in-vitro* exposure (McConnell *et al*., 1995a and b; Adair and McNulty, 1997; Adair, 2000). McConnell *et al*. (1995a and b) showed that Interleukin-1 production, crystalisable fragment (Fc) receptor expression, phagocytosis, and bactericidal activity were all adversely affected, and functional defects were observed in macrophages obtained from birds as from 7 to 42 days after infection. Also, the immunosuppressive effect of chicken infectious anaemia has been associated with minimized vaccinal response and increased pathogenicity of other co-infecting diseases such as IBD (Hagood *et al*., 2000).

2.1.9 Mode of transmission of chicken infectious anaemia virus

Chicken infectious anaemia virus spreads both vertically and horizontally in chickens (Yuasa and Yoshida, 1983; McNulty, 1991; Hoop, 1992). Horizontal transmission occurs through contact with infected chickens, contaminated houses, formites, personnel, etc. Chicken infectious anaemia virus is excreted in the faeces at relatively high titres for several weeks after infection (Yuasa *et al*., 1983b; Hoop, 1992). Davidson *et al*. (2008) carried out an experiment to determine whether the feather shafts are a source of infective virus, the feather shafts were homogenized and used to infect 1-day-old chicks via the mucosal entries (eyes, nose and oropharynx). They demonstrated the CIAV-infection by serology, virology and pathology, showing that feather shafts carry infectious CIAV either on their surface or within their feather pulp, and concluded that feathers contribute to the horizontal dissemination of CIAV. It has been demonstrated experimentally that infected semen transmits CIAV effectively to hens; this could be an important mechanism in primary infections in breeding flocks (Hoop, 1993). Breeder flocks that come into egg production without exposure to CIAV during their growing period usually transmit CIAV vertically to their progeny (Yuasa and Yoshida, 1983). Horizontal spread occurs through ingestion of material contaminated with infected faeces (McNulty, 1991).

2.1.10 Diagnosis of chicken infectious anaemia

A presumptive diagnosis of chicken infectious anaemia can be made based on the signs and lesions in affected birds. However, the presence of anaemia and haematopoietic organ atrophy alone is not sufficient to diagnose the condition (Goodwin and Brown, 1992). A definitive diagnosis can be made by isolation of CIAV, or demonstrating the presence of virus-specific antigens or nucleic acid in affected birds (Todd *et al*., 2001). Immunoperoxidase staining has been described using fixed, paraffin-embedded tissues (Hoop and Reece, 1991; McNeilly *et al*., 1991; Smyth *et al*., 1993). In addition, CIAV antigens have been detected by immunofluorescence using acetone- fixed tissue impression smears or cryostat sections, and a variety of polyclonal antisera and monoclonal antibodies (McNulty *et al*., 1990 a,b; Hoop and Reece, 1991; McNeilly *et al*., 1991). Diagnostic methods successfully used to detect CIAV-specific DNA include dot blot hybridization assays (Noteborn *et al*., 1992), *in-situ* hybridization using paraformaldehyde-fixed blood smears (Novak and Ragland, 1997; Sander *et al*., 1997) and in formalin fixed, paraffin-embedded (FFPE) sections of thymus (Allan *et al*., 1993; Nielsen *et al*., 1995), and PCR performed with chicken tissues and sera, FFPE sections, and vaccines (Noteborn *et al*., 1992; Tham and Stanislawek, 1992; Todd *et al*., 1992; Soinie *et al*., 1993; Rodenberg *et al*., 1994; Dren *et al*., 1994; Goodwin *et al*., 1996; Imai *et al*., 1998). Antibodies to CIAV have been detected using indirect immunofluorescence and immunoperoxidase tests, virus neutralization, and ELISA (McNulty, 1998). The ability to automate this procedures, the short time required to generate results, flexibility, and cost makes ELISA the preferred method for flock screening for CIAV (Todd *et al*., 1999).

2.1.11 Prevention and control of chicken infectious anaemia

The key to preventing outbreaks of chicken infectious anaemia is to ensure that breeder flocks develop antibodies to CIAV before they come into lay. The immunity resulting from exposure to the virus prevents subsequent vertical transmission. Fortunately, the ubiquity of CIAV, its resistance to inactivation by disinfectants and environmental factors, and lapses in biosecurity ensure that primary infection and seroconversion occur in the vast majority of breeder flocks during the rearing period. The same outcome can be achieved by vaccination against CIA (Todd *et al*., 2001). Also, autogeneous vaccine has been used by chicken breeders by using a homogenate prepared from tissues of affected chickens and administered via the drinking water to breeder flocks during the rearing period. A chicken embryo-propagated vaccine, administered via the drinking water to 13 to 15 week old breeders at a dose of $10^{4.5}$ $TCID_{50}$ per bird has also been used (Vielitz *et al*., 1987). An attenuated, live CIAV vaccine, administered by parenteral routes is commercially available (Steenhuisen *et al*., 1994). As CIAV exerts many of its pathogenic effects by acting in concert with other pathogens, it is important to maintain a high level of biosecurity to minimize exposure, coupled with appropriate specific measures against the other pathogens, including vaccination. There is no specific treatment for flocks affected with anaemia dermatitis syndrome. Treatment with broad-spectrum antibiotics has been used to control secondary bacterial infections, but there are no reliable data on its effectiveness (Todd *et al*., 2001).

2.2 Infectious Bursal Disease

2.2.1 Definition of infectious bursal disease

Infectious bursal disease or Gumboro disease is a highly contagious viral infection of young chickens which affects the immune system of poultry and is characterized by the destruction of the lymphoid organs especially the bursa of Fabricius which results in immunosuppression in non fatal cases (Van den Berg *et al*., 2000).

2.2.2 Aetiologic agent of infectious bursal disease

The infectious bursal disease virus (IBDV) belongs to the genus *Avibirnavirus* and family Birnaviridae. The IBDV is a non enveloped icosahedral virus consisting of a genome of two segments of double-stranded ribonucleic acid (Kibenge *et al*., 1988; Van den Berg, 2000). Two serotypes of IBDV have been described which are serotype 1 and serotype 2. Serotype 1 is pathogenic for poultry and serotype 2 is apathogenic and both are differentiated *in-vitro* by lack of cross neutralization and *in-vivo* by lack of cross-protection (McFerran *et al*., 1980; Becht *et al*., 1988).

2.2.3 Structure of infectious bursal disease virus

The IBD viral proteins VP2 and VP3 are structural proteins that form the viral capsid. The VP2 contain the epitopes responsible for the induction of neutralizing and protective antibodies and monoclonal antibodies have been prepared against the VP2 protein (Azad *et al*., 1987; Bayliss *et al*., 1990; Van der Marel *et al*., 1990). The neutralising monoclonal antibodies are serotype-specific while the non-neutralising antibodies act against VP2 or VP3 (Jackwood *et al*., 1984; Oppling *et al*., 1991).

2.2.4 Incidence and distribution of infectious bursal disease

Cosgrove in the area of Gumboro in Delaware was the first to report infectious bursal disease in 1962 (Cosgrove, 1962). Infectious bursal disease was common in America between 1960 to 1964 (Lasher and Davis, 1997). Infectious bursal disease later spread to Europe from 1962 to 1971 (Faragher, 1972). Infectious bursal disease was detected between 1966 and 1974 in the middle east, south and western Africa, India, the far east and Australia (Faragher, 1972; Firth, 1974; Jones, 1986; Van der Sluis, 1999). Infectious bursal disease is currently distributed worldwide (OIE, 1995).

2.2.5 Pathology and lesions caused by infectious bursal disease virus

The IBDV mainly affects the bursa of Fabricius although other lymphoid organs are affected (Tanimura and Sharma, 1997). The IBDV causes cytolysis of actively dividing B-lymphocytes (Burkhardt and Müller, 1987). The B-lymphocytes are susceptible at their immature stage to IBDV and immunoglobulin M is carried on the B-lymphocytes surfaces resulting in an absurd immune response in which immunosuppression co-exist with high anti-IBDV antibody titres (Van den Berg, 2000).

The macroscopic lesions seen in the bursa of Fabricius, three to four days after infection exhibit inflammatory signs which are hypertrophic, hyperaemic and oedematous bursa. The above lesions are seen three to four days following infection. At the fifth day, reversion of the bursa to its normal size occurs and reversion to less than one third its size in the eight day. Hypertrophic and whitish kidneys that contain deposits of urate crystals have been reported (Van den Berg, 2000). Haemorrhagic pectoral and thigh muscles which is likely caused by coagulation disorders are sometimes seen (Skeeles *et al*.,1980). In the acute form of IBD macroscopic lesions are seen in other lymphoid organs such as the spleen and thymus (Nicholas *et al*., 1985; Hiraga *et al*.,1994; Inoue *et al*., 1994; Inoue *et al*., 1999).

2.2.6 Clinical signs of infectious bursal disease

Infectious bursal disease has an incubation period of 2 to 3 days and birds show exhaustion, prostration, dehydration, have watery diarrhoea and ruffled feathers (Van den Berg, 2000). On day three, mortality begins in affected flocks, peaks on the fourth day, the remaining surviving birds show recovery after the fifth to seventh day. Four factors influence the severity of IBD which are age, breed sensitivity, virulence of IBDV strain and the degree of passive immunity (Van den Berg, 2000).

2.2.7 Clinical forms of infectious bursal disease

The classical form of IBD was described in the early 1960s and is caused by the classical virulent form of IBDV. It is characterized by a low specific mortality, a mostly subclinical disease that occurs when there is a reduction in the level of passive antibodies (Faragher, 1972). The immunosuppressive form was seen in the United States of America and its aetiology is the classical low pathogenicity strains of IBDV and variant strains that resist neutralization by antibodies against classical viruses (Jackwood and Saif, 1987; Snyder, 1990). The acute form was described in Europe and Asia with aetiology of hypervirulent strains of IBDV which results in high mortality (Van den Berg *et al*., 1991).

2.2.8 Pathogenesis and immunosuppression caused by infectious bursal disease

Chickens acquire IBDV by ingestion under natural conditions. The IBDV target the IgM+ cells. Replication of the IBDV occurs widely in the bursa of Fabricius. Within the acute phase of the IBD which last for about 7 to 10 days, bursal follicles experience depletion of their B-cells and the bursa atrophies. At this point abundant viral antigens are detectable in the bursal follicles and other peripheral lymphoid organs such as the caecal tonsils and spleen. CD4 (+) and CD8 (+) T cell accumulate at and near the site of virus replication. The virus-induced bursal T cells are activated, exhibit upregulation of cytokine genes, proliferate in response to *in-vitro* stimulation with IBDV and have suppressive properties. Chickens may die during the acute phase of IBD although IBDV induced mortality is highly variable and depends, among other factors, upon the virulence of the virus strain. Chickens that survive the acute IBD clear the virus and recover from its pathologic effects. Bursal follicles are repopulated with IgM (+) B cells. Clinical and subclinical infection with IBDV may cause immunosuppression. Both humoral and cellular immune responses are compromised. Humoral immunity inhibition is due to destruction of immunoglobulin producing cells by IBDV. Alteration of antigen-presenting and helper T cell functions may also be involved. Infectious bursal disease virus infection also causes a short lived inhibition of the *in vitro* proliferative response of T cells to mitogens. The inhibition is brought about by macrophages that get activated in chickens exposed to virus (Sharma *et al*., 2000).

2.2.9 Protection against infectious bursal disease virus

Protection against IBD is mainly through humoral immunity and a close correlation exists between antibody titres and protection (Van den Berg, 2000). Maternally derived antibodies passively protect chicks against IBD (Van den Berg, 2000). A knowledge of the maternally derived antibody titre at hatch enables one to predict when most of the flock will be vulnerable to the field virus or vaccinal virus which helps in arriving at a time for vaccination (DeWit, 1999).

2.2.10 Susceptibility factors to infectious bursal disease virus

Birds of between 3 to 6 weeks of age are most susceptible to IBD. Infections that occur before 3 weeks of age are subclinical and immunosuppressive but birds of 15 to 20 weeks have shown clinical signs. The light weight layer strains are more susceptible to IBD than heavy weight broiler strains (Hassan and Saif, 1996).

2.2.11 Susceptible hosts to infectious bursal disease

The chicken is the only susceptible host to serotype 1 IBDV while the turkey is an asymptomatic carrier of serotype 2. Other domestic birds are asymptomatic carriers of serotypes 1 and 2 and wild birds act as reservoirs and vectors (Jackwood *et al*., 1982; Wilcox *et al*., 1983).

2.2.12 Mode of transmission of infectious bursal disease virus

The IBDV is transmitted only horizontally through the oral route and respiratory tract. The IBDV is excreted in the faeces of infected birds. Direct contact with excreting subjects and indirect contact with contaminated objects or living vectors are ways in which IBDV is transmitted (Van den Berg, 2000).

2.2.13 Diagnosis of infectious bursal disease

2.2.13.1 Clinical diagnosis

Clinical diagnosis is based on manifestation of IBD in which there is a peak in mortality with recovery in 5 to 7 days, observation of symptoms and postmortem findings (Van den Berg, 2000).

2.2.13.2 Differential diagnosis

Diseases that can be mistaken for IBD are avian coccidiosis, Newcastle disease, stunting syndrome, chicken infectious anaemia, mycotoxicosis and nephropathogenic forms of infectious bronchitis (Van den Berg, 2000).

2.2.13.3 Methods of diagnosis

Histological diagnosis is based on detecting modifications in the bursa. Serological test detect antibodies. Examples of serological tests are agar gel immune diffusion, enzyme linked immunosorbent assay and serum neutralization. Virological diagnosis detects the IBDV in the bursa of Fabricius in the acute phase of the disease (Van den Berg, 2000).

2.2.14 Prevention and control of infectious bursal disease

Biosecurity measures and medical prophylaxis in combination with vaccination is used to prevent IBD. The practice of all-in/all-out farming method, cleaning and disinfection of premises are precautionary sanitary methods used to prevent IBD. Also, in the practice of all-in /all-out method of farming, elimination of rodents and insects must be carried out when the farm is empty. The type of vaccines used against IBD are attenuated live virus vaccines and oil emulsion inactivated virus vaccines. The live vaccines are of three types mild, intermediate and hot (Van den Berg, 2000).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Area

The study was carried out in Zaria which is located in Kaduna State and situated in the Northern Guinea savannah zone of Nigeria between Latitude 6° and 11° 3´ N and Longitude 7° and 44° E. The climate of Zaria is tropical continental, characterized by cool humid wet season and cold or hot dry season. The average rainfall ranges from 1000 mm to 1250 mm. The average daily temperature ranges from 17-33 °C (Mortimore, 1970). As at 2003, Kaduna State had a total poultry population of 2,821,093; consisting of 2,564,100 backyard poultry and 256,993 exotic poultry (Adene and Oguntade, 2006). Kaduna State has an area of about 48,473.23 sq Km, with a human population of over 6,060,562 people according to the census figures of 2006 (KDSG, 2008).

3.2 Determination of Sample Size

Sample size was determined using the formular given by Michael (1995)

$$n = \frac{1.96^2 \times Pexp \times (1-Pexp)}{d^2}$$

A chicken infectious anaemia seroprevalence of 83% reported by Owoade *et al.* (2004) was used.

where n = required sample size

Pexp = expected prevalence (83%= 0.83)

d = desired absolute precision (5%= 0.05)

Therefore minimum sample size, n = $1.96^2 \times 56.44 = 218$ serum samples.

Purposive sampling was used to collect the samples.

3.3 Materials

3.3.1 Chicken anaemia antibody test kit and infectious bursal disease antigen and antiserum

Chicken anaemia virus antibody test kits (IDEXX Laboratories, Inc, U.S.A), QIAamp DNA mini kit, dNTP, and primers were obtained from Inqaba, South Africa through the National veterinary institute, Vom. Infectious bursal disease positive antiserum and antigen were provided by Prof. Paul Abdu of the Department of Veterinary Medicine, Ahmadu Bello University, Zaria.

3.3.2 QIAamp DNA Mini Kit

The kit is for DNA extraction and contains QIAamp Mini Spin Columns, QIAGEN proteinase k, reagents, buffers, collection tubes (2 ml) (Qiagen, Netherlands).

3.3.3 Forward and reverse primers

Specific primers for CIAV were, forward primer: CAV1 (5' GCA GTA GGT ATA CGC AAG GC 3') and reverse primer: CAV2 (5' CTG AAC GTT GAT GGT C 3') that covers a 186-bp region on the highly conserved VP2 coding gene.

3.4 Collection of Samples

3.4.1 Collection of blood samples

Blood samples (351) were collected from chickens of ages between 1 day old to 45 weeks in Zaria and environs. The locations of sampling were the Avian Clinic of the Veterinary Teaching Hospital of Ahmadu Bello University Zaria, a private veterinary clinic in Palladan-Zaria, Samaru live bird market and Sabon gari live bird market. Blood was collected through the jugular vein or the heart using 2 ml syringes with 23 G needles or 5 ml syringes with 21 G needles, depending on the size of the chicken. Chickens that were sampled included broilers, layers and breeders. The blood collected from each bird was allowed to clot in the syringes at room temperature for at least 2 hours to separate the serum. The sera were decanted into 5 ml sample bottles. The sera were stored in a deep freezer (at -20°C) until they were tested for CIA and IBD antibodies. The Veterinary Teaching Hospital Avian clinic record sheet template (Appendix 1) was used to record data about each flock presented as a clinical case.

3.4.2 Collection and processing of bursa of Fabricius and thymus

Bursa of Fabricius and thymus of sick or dead chickens from flocks suspected of having IBD outbreak were removed and stored in a deep freezer at -20°C. The bursae and thymus were later ground using a mortar and pestle. A measured quantity of phosphate buffered saline (PBS) with a pH of 7.4 was added to each ground bursa or thymus at the ratio of 1 ml to each 1 gram and centrifuged at 3000 rpm for 30 minutes. The supernatant was decanted into sample bottles and stored in the deep freezer (at -20°C) before carrying out agar gel immunodiffusion (AGID) test for infectious bursal disease antigen and PCR for CIAV VP2 gene.

3.5 Laboratory Tests

3.5.1 Polymerase chain reaction

3.5.1.1 Deoxyribonucleic acid extraction

20 µl of QIAGEN protease (proteinase K) was dispensed into 1.5 ml centrifuge tubes. 200 µl of each processed bursal and thymic sample was added to the microcentrifuge tubes. 200 µL buffer AL (lysis buffer) was added to samples and mixed by pulse vortexing for 15 seconds, They were incubated at 56 °C for 10 minutes. 200 µL of ethanol (between 96-100%) was added to the samples and mixed again by pulse vortexing for 15 seconds. The 1.5 ml microcentrifuge tube was briefly centrifuged at 8,000 rpm for 10 sec (at 6,000 g for 10 seconds) to remove drops from the inside of the lid. The mixture was carefully applied to QIAamp Mini spin columns (in 2 ml collection tubes). The caps were closed and centrifuged at 6,000 g (8,000 rmp) for 1 minute. The QIAamp Mini spin columns were placed in 2 ml collection tubes and the collection tubes containing the filtrate were discarded. The QIAamp Mini spin columns were carefully open and 500 µL buffer AW1(wash buffer) was added. The caps were closed and the content were centrifuged at 6,000 g (8,000 rpm) for 1 minute. The QIAamp Mini spin columns were placed in clean 2 ml collection tubes and the collection tube containing the filtrate were discarded. The QIAamp Mini spin columns were carefully opened and 500 µL buffer AW2(wash buffer) was added. The caps were closed and the contents were centrifuged at full speed 20,000 g (14,000 rpm) for 3 minutes. The QIAamp Mini spin columns were placed in clean 1.5 ml microcentrifuge tubes and the collection tube containing the filtrate were discarded. The QIAamp Mini spin column were opened and 60 µL buffer AE (elution buffer) was added. The extracted DNA were incubated at room temperature 15-25 °C for 1 minute and was then centrifuged at 6,000 g (8,000 rpm) for 1 minute.

3.5.1.2 Master mix preparation for chicken infectious anaemia virus polymerase chain reaction

The gene Amp core kit (Applied biosystem, U.S.A) was used to prepare the master mix. The master mix was a 50 µL reaction volume consisting of 2 µL of isolated DNA, 5 µL of 10X reaction buffer (Tris-hydrochloric acid), 1 µL of 10 Mm deoxynucleoside triphosphate mix, 1.5 µL of 25 Mm $MgCl_{2,}$ 0.5 µL of 5 U/µL of Platinum *Taq* DNA polymerase (Promega, Madison, WI), 1 µL of each primer (20 pmol), and 38 µL of nuclease- free water.

3.5.1.3 Deoxyribonucleic acid amplification

Deoxyribonucleic acid amplification was performed in automatic thermocyclers (AB Applied Biosystems verity 96 well thermal cycler and Gene Amp PCR system 9700). The reactions consisted of denaturation for 2 minutes at 94 °C followed by 35 cycles of denaturation for 30 seconds at 94 °C, primer annealing for 30 seconds at 60 °C and extension for 1 minute at 72 °C. After the final 7 minute extension at 72 °C, the samples were held at 4 °C until they were removed from the thermocycler.

3.5.1.4 Agarose gel electrophoresis

A 1% agarose gel was prepared by dissolving agar in the ratio of 1gram agar to 100 ml water. The mixture was heated in a microwave oven (LG) and allowed to cool. Some of the agar was poured into the casting system (BIO RAD) placed on a flat surface. The gel was allowed to thicken and next transferred to an electrophoresis tank (BIO RAD) into which 1x tris-acetate-EDTA buffer was poured. Wells were punched into the gel using a comb. A 3 µl of loading dye (Promega) was added to the DNA sample. The DNA samples were loaded into the wells. The electrophoresis was powered by a power pack (Consort, CV202). Electrophoresis was run for 40 minutes at 120 volts. The PCR product sizes were visualized by UV illumination in 1% agarose gel stained with ethidium bromide.

3.5.2 Serological assay

3.5.2.1 Description and principles of chicken anaemia virus test kit

The IDEXX CAV is an enzyme immunoassay for the detection of antibody to CIAV in chicken serum. The IDEXX CAV assay is performed in a microtitre plate coated with CIAV antigen. During the first incubation, CIA antibodies present in the sample react with immobilized antigens. After a wash step, an Anti-CIAV monoclonal antibody enzyme conjugate is added to the microwell. If no Anti-CIA antibodies are present in the test sample, the Anti-CIAV conjugate is free to react with the CIAV antigen. Conversely, if Anti-CIAV antibodies are present in the sample, the enzyme conjugated monoclonal antibodies are blocked from reacting with the antigen. Following incubation period, the unreacted conjugate is removed by washing with washing buffer and a substrate/chromogen solution is added. In the presence of enzyme, the substrate is converted to a product which reacts with the chromophore to generate a blue colour. The absorbance at 650 nm, A(650), is measured using a spectrophotometer. Results are calculated by dividing the A(650) of the sample by the mean A(650) of the negative control, resulting in an S/N value. The quantity of antibodies to CIAV is inversely proportional to the A(650) and thus, to the S/N value. The presence of CIAV antibodies indicate previous exposure to chicken infectious anaemia virus.

3.5.2.2 *Enzyme linked immunosorbent assay*

Sera were tested using a commercial test kit that detects antibody subsequent to field exposure to CIAV. The commercial test kit, IDEXX CAV antibody test kit was used according to the manufacturers instruction.

All the reagents were allowed to come to between 18-26°C and then mixed by gently inverting and swirling the content. The antigen coated plates were removed from the packet and sample positions recorded. 100 µL of undiluted negative control was dispensed into duplicate wells. 100 µL of undiluted positive control was dispensed into duplicate wells. 100 µL of diluted sera samples (diluted in ten fold, 1/10) were dispensed into wells. The samples in the wells were incubated for 60 minutes at 18-26°C. Each well was washed with approximately 350 µL of washing buffer 3 times. 100 µL of conjugate were dispensed into each well. The wells were incubated for 30 minutes at 18-26° C. Each well was washed with 350 µL of washing buffer 3 times. 100 µL of TMB substrate solution was added into each well and incubated for 15 minutes at 18-26°C. 100 µL of stop solution was dispensed into each well to stop the reaction. Finally, absorbance values at 650 nm, A(650) were measured and recorded. The presence or absence of antibody to CIAV was determined by the sample to negative (S/N) ratio for each sample. S/N Ratio = Sample Absorbance (650)/ mean of negative controls. The results were interpreted as follows: samples of S/N ratios greater than 0.6 were considered negative, samples with S/N ratios less than or equal to 0.6 were considered positive.

3.5.2.3 Agar gel immunodiffusion test

The agar gel was prepared by weighing 4.5 grams of agar, 0.6 grams of sodium azide and 24 grams of sodium chloride into a conical flask. 300 ml of distilled water was added and stirred by swirling. The mixture was heated in the conical flask on a Bunsen burner until complete dissolution of agar. The mixture was stirred while being heated. 25 ml of agar were pipetted and poured into each plate (9 × 9.5) and allowed to spread (12 plates were used). The agar was allowed to set and wells were punched with microtitre tits into the agar using a template. A needle was used to pick out the centre agar to create hollow wells (Harai *et al*., 1972).

Antibodies and antigens of IBD were detected using AGID test (OIE, 2008). To detect IBD antigens, 25 µl of processed bursa of Fabricius that were removed from chickens were placed in peripheral wells against 25 µl positive sera in the central wells and incubated in a humid chamber for 3 days at room temperature. Wells positive for IBD antigen were those with a precipitin line between the central wells containing positive sera and the peripheral wells containing the processed bursal sample (Plate V). Antibodies to infectious bursal disease were detected using the AGID test. The sera were placed in wells against infectious bursal disease virus antigen. 25 µL of sera were placed in the peripheral well against 25 µl of IBD antigen in the central wells and incubated in a humid chamber for 3 days at room temperature (22-30°C). Wells positive for IBD antibody were those with a precipitin line between the central wells containing the IBD antigen and the peripheral wells containing the sera.

3.6 Data Analyses

Chi-square was used to determine association between chicken infectious anemia antibodies presence in flocks and infectious bursal disease antibodies. Chi-square was determined using statistical packages for social sciences (SPSS) version 16 and graph pad prism version 5.03. Odd ratios were calculated using winepiscope and graph pad prism version 5.06. A 95% confidence interval was used and results with p value less than 0.05 were considered significant. Age distribution of chicken infectious anaemia antibodies was carried out by placing chickens sampled in to age groups 0-6 weeks, 7-18 weeks and >19 weeks. Odds ratio was used to compare 7-18 weeks old and > 19 weeks old to 0-6 weeks old. Odd ratio with p value less than 0.05 were considered significant. Clinical signs and pathologic lesions were expressed in percentages.

CHAPTER FOUR

RESULTS

4.1 Number of Samples Collected

In this study, a total of 351 sera, 69 bursae of Fabricius and 63 thymus were collected from April 2013 to April 2014 (Appendix 2).

4.2 Clinical Signs of Chickens that Tested Positive for Chicken Infectious Anaemia Antibodies

Some chickens were positive for antibodies to chicken anaemia virus. Some of the seropositive chickens showed paleness as a clinical sign, unwillingness to move, drooped wings, ruffled feathers, uneven growth and weight loss (Table 4.1).

4.3 Clinical Signs of Chickens with Infectious Bursal Disease Virus

The clinical signs exhibited by chickens that tested positive for IBD antigen were listlessness, sitting on the hock, in-coordinated gait, depression, sudden death, ruffled feathers, somnolence and weakness (Table 4.2).

Table 4.1:Clinical signs of chickens that tested positive for chicken infectious anaemia antibodies in Zaria and environs, Nigeria using enzyme linked immunosorbent assay.

	Clinical signs					
Flock / No.	Paleness	Unwilling to move	Drooped wings	Ruffled feathers	Uneven growth	Weight loss
4	-	+	-	+	-	-
6	-	-	-	-	-	-
7	-	-	-	-	-	-
8	-	-	-	-	-	-
9	-	-	-	-	-	-
10	-	-	-	-	-	-
12	-	+	+	-	-	-
16	-	+	-	+	-	-
17	+	-	-	-	-	+
18	-	-	-	+	+	+
20	+	-	-	-	-	+
23	-	+	-	-	-	-
24	-	+	-	-	-	-
26	-	-	-	-	-	-
28	-	-	-	-	-	-
30	-	-	-	-	-	-

31	-	-	-	-	-	-
32	-	-	-	-	-	-
33	-	-	-	-	-	-
34	-	-	-	-	-	-
38	-	-	-	-	-	-
39	-	+	+	-	-	-
41	-	-	-	-	-	-
44	-	-	-	-	-	-
47	-	-	-	-	-	-
51	+	+	-	+	-	-
55	-	-	-	-	-	-

Key

+ = presence of clinical sign

- = absence of clinical sign

Table 4.2: Clinical signs of chickens that tested positive for infectious bursal disease virus in Zaria and environs, Nigeria using agar gel immunodiffusion test.

	Clinical signs							
Flock No.	Listlessness	Sitting on the hock	In-coordinated gait	Depression	Sudden death	Ruffled feathers	Somnolence	Weakness
22	-	-	-	-	+	-	-	-
45	-	-	-	-	-	+	+	+
52	-	-	-	-	+	-	-	-
57	-	-	+	+	-	-	-	-
58	+	+	-	-	-	+	+	-

Key

+ = presence of clinical signs

- = absence of clinical signs

4.4 Chicken Infectious Anaemia Antibodies

Out of the 351 sera tested for CIA antibodies using ELISA, 207 (58.97% which is the overall prevalence) tested positive. The sera were obtained from 47 flocks, out of this number, 27 (57.4%) tested positive for CAV antibodies representing flock prevalence (Table 4.3). Positive samples were those with absorbance of less than 0.5 (Plate IV).

4.5 Infectious Bursal Disease Antibodies

Out of 346 sera tested for IBD antibodies, 115 (33.2%) were positive representing overall prevalence. The sera were obtained from 47 flocks, out of which 23 flocks (48.9%) tested positive for IBD antibodies also representing flock prevalence (Table 4.4). Positive samples were those that produced a white precipitin line (Plate V).

4.6 Presence of Chicken Infectious Anaemia Antibodies and Infectious Bursal Disease Antibodies in the Same Flock

Cross tabulation (Table 4.5) showed that 18/47 (38.3%) flocks had both chicken infectious anaemia and infectious bursal disease antibodies. It also showed that 6/47 (12.8%) flocks were positive only for infectious bursal disease antibodies, while 9/47 (19.1%) were positive only for chicken infectious anaemia antibodies and 14/47 (29.8%) flocks were both negative for chicken infectious anaemia and infectious bursal disease antibodies. The Chi square (6.182) showed that there was association between presence of chicken infectious anaemia antibodies and infectious bursal disease antibodies in the flocks, and was statistically significant at 0.05 level (Table 4.5).

Table 4.3: Seroprevalence of chicken infectious anaemia in Zaria and environs, Kaduna State, Nigeria

S/No.	Flock Identity	Type of chicken	Age of chicken (weeks)	No. of birds tested for chicken infectious anaemia antibodies	No. of positive sera	Per cent positive sera
1	1	Pullet	7	4	0	0
2	3	Broiler	3	1	0	0
3	4	Pullet	15	3	2	66.67
4	5	Pullet	5	2	0	0
5	6	Broiler	7	1	1	100
6	7	Layer	20	12	8	66.67
7	8	Layer	28	10	9	90
8	9	Broiler Breeder	45	93	86	92.47
9	10	Layer	20	20	16	80
10	11	Layer	20	20	9	45
11	12	Broiler	1	1	1	100
12	16	Broiler	2	2	0	0
13	17	Pullet	11	1	1	100

14	18	Pullet	14	16	10	62.5
15	20-1	Broiler	3	1	0	0
16	20	Layer	19	1	1	100
17	23	Broiler	1	2	2	100
18	24	Broiler	<1	3	2	66.67
19	SM	Broiler	8	2	1	50
20	SM	Broiler	7	1	0	0
21	28	Layer	32	1	1	100
22	SM	Broiler	8	1	0	0
23	SM	Broiler	8	3	1	33.33
24	SM	Broiler	10	9	4	44.44
25	SM	Broiler	8	8	6	75
26	SM	Broiler	8	17	10	58.82
27	SM	Broiler	8	7	6	85.71
28	35	Layer	21	8	0	0
29	36	Broiler	4	2	0	0
30	37	Layer	6	2	0	0
31	SG	Broiler	8	32	20	62.5
32	39	Pullet	14	1	1	100
33	40	Broiler	4	1	0	0
34	SM	Broiler	8	8	2	25
35	SM	Broiler	8	12	2	16.67
36	45	Pullet	4	1	0	0
37	47	Pullet	7	1	0	0
38	SM	Broiler	8	8	1	12.5
39	48	Broiler	5	1	0	0
40	49	Broiler	3	1	0	0
41	51	Pullet	16	4	3	75
42	53	Broiler	7	1	0	0

43	54	Broiler	10	1	0	0
44	55	Broiler	10	5	1	20
45	56	Broiler	10	2	0	0
46	57	Pullet	3	2	0	0
47	58	Broiler	2	16	0	0
Overall prevalence				351	207	58.97

SM Samaru live bird market

SG Sabon gari live bird market

Table 4.4: Seroprevalence of infectious bursal disease in Zaria and environs, Kaduna State, Nigeria.

S/No.	Flock identity	Type of chicken	Age of chicken	No. of birds tested	No. of positive sera (%)
1	1	Pullet	7	3	0 (0.00)
2	3	Broiler	3	1	0 (0.00)
3	4	Pullet	15	3	0 (0.00)
4	5	Pullet	5	2	0 (0.00)
5	6	Broiler	7	1	0 (0.00)
6	7	Layer	20	12	7 (58.33)
7	8	Layer	28	9	6 (66.67)
8	9	Broiler Breeder	45	91	24 (26.37)
9	10	Layer	20	20	2 (10.00)
10	11	Layer	20	20	1 (5.00)
11	12	Broiler	1	1	0 (0.00)
12	16	Broiler	2	2	0 (0.00)
13	17	Pullet	11	2	0 (0.00)
14	18	Pullet	14	16	2 (12.50)
15	20-1	Broiler	3	1	0 (0.00)
16	20	Layer	19	1	0 (0.00)
17	23	Broiler	1	1	0 (0.00)
18	24	Broiler	<1	3	0 (0.00)
19	SM	Broiler	8	2	2 (100.00)
20	SM	Broiler	7	1	0 (0.00)
21	28	Layer	32	1	0 (0.00)
22	SM	Broiler	8	1	1 (100.00)
23	SM	Broiler	8	3	2 (66.67)
24	SM	Broiler	10	10	6 (60.00)
25	SM	Broiler	8	8	1 (12.50)
26	SM	Broiler	8	17	3 (17.65)
27	SM	Broiler	8	7	2 (28.57)
28	35	Layer	21	8	6 (75.00)
29	36	Broiler	4	2	0 (0.00)
30	37	Layer	6	2	1 (50.00)

31	SG	Broiler	8	32	21 (65.63)
32	39	Pullet	14	1	0 (0.00)
33	40	Broiler	4	1	0 (0.00)
34	SM	Broiler	8	8	4 (50.00)
35	SM	Broiler	8	12	1 (8.33)
36	45	Pullet	4	1	0 (0.00)
37	47	Pullet	7	1	1 (100.00)
38	SM	Broiler	8	8	2 (25.00)
39	48	Broiler	5	1	0 (0.00)
40	49	Broiler	3	1	0 (0.00)
41	51	Pullet	16	4	2 (50.00)
42	53	Broiler	7	1	0 (0.00)
43	54	Broiler	10	1	0 (0.00)
44	55	Broiler	10	4	4 (100.00)
45	56	Broiler	10	2	2 (100.00)
46	57	Pullet	3	1	0 (0.00)
47	58	Broiler	2	16	12 (75.00)
Overall seroprevalence				346	115 (33.2)

Table 4.5: Presence of chicken infectious anaemia antibodies and infectious bursal disease antibodies in flocks from Zaria and environs, Nigeria from year 2013-2014.

	Presence of CIA antibodies (%)	Absence of CIA antibodies (%)	Total (%)
Presence of IBD antibodies (%)	18 (38.3)	6 (12.8)	24 (51.1)
Absence of IBD antibodies (%)	9 (19.1)	14 (29.8)	23 (48.9)
Total (%)	27 (57.4)	20 (42.6)	47 (100)

X^2 (Yates correction) = 4.8 > 3.84 p = 0.0129

Key

CIA= chicken infectious anemia

IBD = infectious bursal disease

4.7 Detection of Chicken Infectious Anaemia and Infectious Bursal Disease Antibodies in Chickens

The odds of detecting antibodies to chicken infectious anaemia compared to infectious bursal disease antibodies was 2.89 and was statistically significant with a p value < 0.0001 (Table 4.6). This shows that the ratio of detection of chicken infectious anaemia to infectious bursal disease antibodies in Zaria was 2.89:1. The chi square (1.823) showed that there was no association between presence of both chicken infectious anaemia and infectious bursal disease antibodies in chickens (Table 4.6).

4.8 Infectious Bursal Disease Antigens

Of the 69 bursa of Fabricius tested, 16 (23.2%) were positive for IBD virus. The individual prevalence was 23.2%. Of the 29 flocks tested for IBD viruses, 11 flocks (37.9%) tested positive. The flock prevalence ranged from 15.38-100% (Table 4.7).

4.9 Chicken Infectious Anaemia Virus

Thymus from twenty six flocks were tested for chicken infectious anaemia virus antigen using PCR, 3.8% (1/26) of the flocks were positive. A total of 63 thymus were tested and 3.17% (2/63) were positive using PCR (Plate I and II) (Table 4.8). Bursae from twenty eight flocks were tested for chicken infectious anaemia virus and 3.57% (1/28) of the flocks tested positive. A total of 67 bursae were tested and 1.49% (1/67) were positive for chicken infectious anaemia virus using PCR (Plate III).

Table 4.6: Presence of both chicken infectious anaemia antibodies and infectious bursal disease antibodies in individual chickens sampled in Zaria and environs, Nigeria from year 2013-2014.

Antibody	No of samples Tested	Number positive	Number negative	Odds ratio (OR)
CIAab	351	207	144	2.89
IBDab	346	115	231	1.00
Total	697	322	375	

Chi square = 1.823
P < 0.1769

OR= 2.89

CI = 2.121 to 3.932
P= 0.0001

Key

CIA = chicken infectious anaemia

IBD = infectious bursal disease

ab = antibody

Table 4.7: Prevalence of infectious bursal disease in Zaria and environs, Kaduna State, Nigeria using agar gel immunodiffusion test.

S/No.	Flock identity	Type of chicken	Age of chicken (weeks)	No. of birds tested	No. of positive bursa	Percent positive for each flock (%)
1	1	Pullet	3	2	1	50.00
2	2	Pullet	4	2	1	50.00
3	3	Pullet	3	2	1	50.00
4	4	Broiler	5	3	1	33.33
5	13	Broiler	2	2	0	0.00
6	14	Pullet	3	3	0	0.00
7	15	Broiler	7	1	0	0.00
8	16	Broiler	2	1	0	0.00
9	19	Broiler	4	2	0	0.00
10	20	Broiler	3	1	0	0.00
11	21a	Pullet	3	2	0	0.00
12	21b	Broiler	4	4	0	0.00
13	22	Broiler	4	2	2	100.00
14	23	Broiler	1	1	0	0.00
15	25	Broiler	3	3	0	0.00
16	37	Pullet	6	2	0	0.00
17	40	Broiler	4	1	0	0.00
18	42	Broiler	7	1	0	0.00
19	43	Broiler	3	3	2	66.67
20	45	Pullet	4	13	2	15.38
21	46	Pullet	2	3	0	0.00
22	48	Broiler	5	1	0	0.00
23	49	Broiler	3	1	0	0.00
24	50	Broiler	5	2	1	50.00
25	52	Pullet	3	4	3	75.00
26	54	Broiler	5	1	0	0.00
27	57	Pullet	3	3	1	33.33
28	58	Broiler	3	1	1	100.00
29	59	Broiler	5	2	0	0.00
Overall prevalence				69	16	23.20

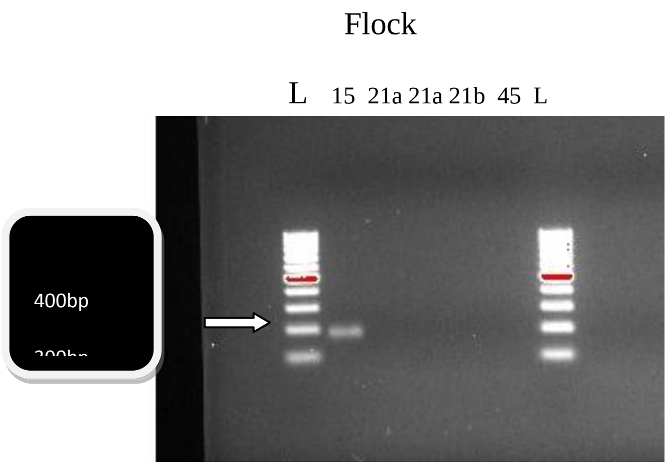

Plate I: Visualization of polymerase chain reaction band after electrophoresis with ultraviolet light illumination. L (ladder), flock 15 thymus was PCR positive for chicken infectious anaemia virus. Arrow shows 186 bp which is specific for CIA antigen. Type of bird was 7 weeks old broiler.

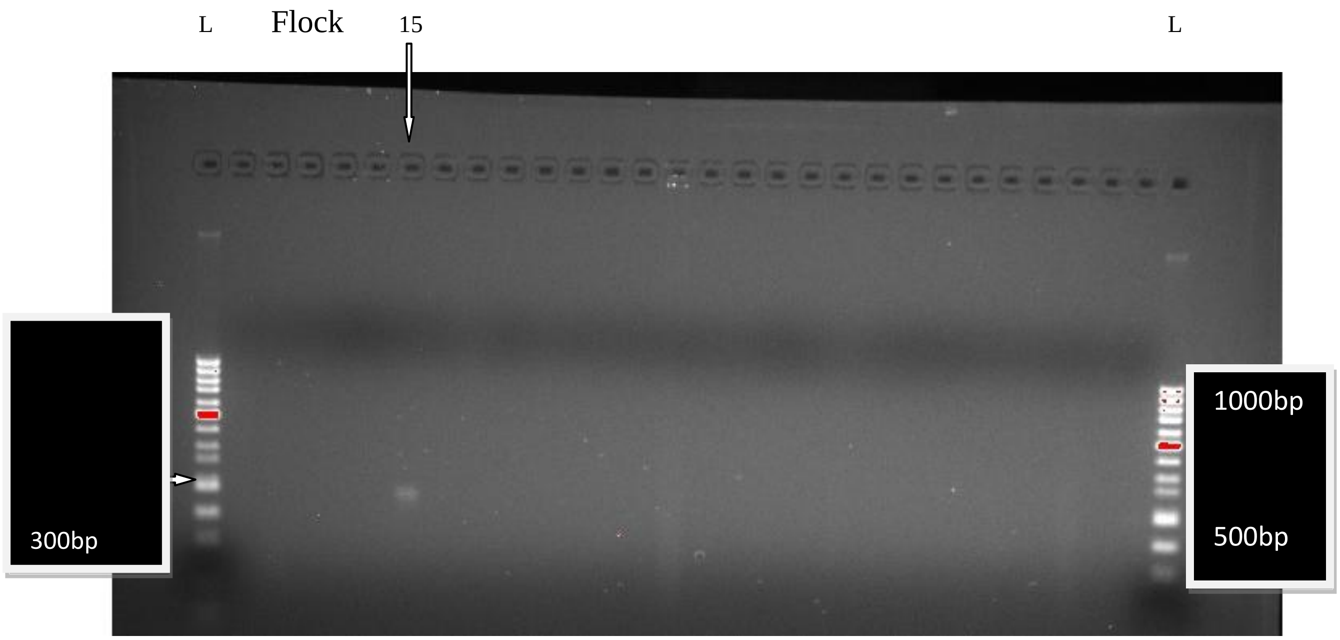

Plate II: Visualization of polymerase chain reaction product with ultraviolet light in ethidium bromide stained agarose gel. The plate shows another thymus from 7 weeks old broiler in flock 15 PCR positive. Vertical arrow shows positive lane for flock 15. Horizontal arrow shows 186 bp which is specific for CIA viruses. L (Ladder).

Table 4.8: Prevalence of chicken infectious anaemia in sampled flocks using polymerase chain reaction.

Flock No.	No. of thymus tested	No. of thymus positive (%)	No. of bursa tested	No. of bursa positive (%)
1	0	0 (0)	2	0 (0)
2	0	0 (0)	1	0 (0)
3	0	0 (0)	3	0 (0)
4	0	0 (0)	1	1 (100)
12	1	0 (0)	0	0 (0)
13	0	0 (0)	2	0 (0)
14	4	0 (0)	3	0 (0)
15	2	2 (100)	1	0 (0)
16	2	0 (0)	1	0 (0)
19	2	0 (0)	2	0 (0)
20	1	0 (0)	1	0 (0)
21a	2	0 (0)	2	0 (0)
21b	4	0 (0)	4	0 (0)
22	2	0 (0)	2	0 (0)
23	3	0 (0)	1	0 (0)
25	3	0 (0)	3	0 (0)
34	2	0 (0)	2	0 (0)
37	2	0 (0)	0	0 (0)
40	1	0 (0)	1	0 (0)
42	1	0 (0)	1	0 (0)
43	3	0 (0)	3	0 (0)
45	11	0 (0)	13	0 (0)
46	2	0 (0)	3	0 (0)
48	1	0 (0)	1	0 (0)
49	1	0 (0)	1	0 (0)
50	2	0 (0)	2	0 (0)
52	4	0 (0)	4	0 (0)
54	1	0 (0)	1	0 (0)
57	2	0 (0)	3	0 (0)
58	1	0 (0)	1	0 (0)
59	2	0 (0)	2	0 (0)
Overall prevalence	63	2 (3.17)	67	1 (1.49)

4.10 Flock Positive for both Chicken Infectious Anaemia And Infectious Bursal Disease viruses

One (3.6%) (1/28) of the bursa from flock 4 tested PCR positive for chicken infectious anaemia virus (Plate III and Table 4.8) and was also positive for infectious bursal disease using agar gel immunodiffusion (Table 4.7).

4.11 Observations on Flock Detection of Infectious Bursal Disease Antibodies and Antigens using Agar Gel Immunodifusion Test

In flock 45 and 57, in which both IBD antibodies and antigens were tested for, it was observed that where antibodies were absent, viruses were detected. However, flock 58 did not follow this pattern as both IBD antigens and antibodies were detected (Table 4.3 and 4.7).

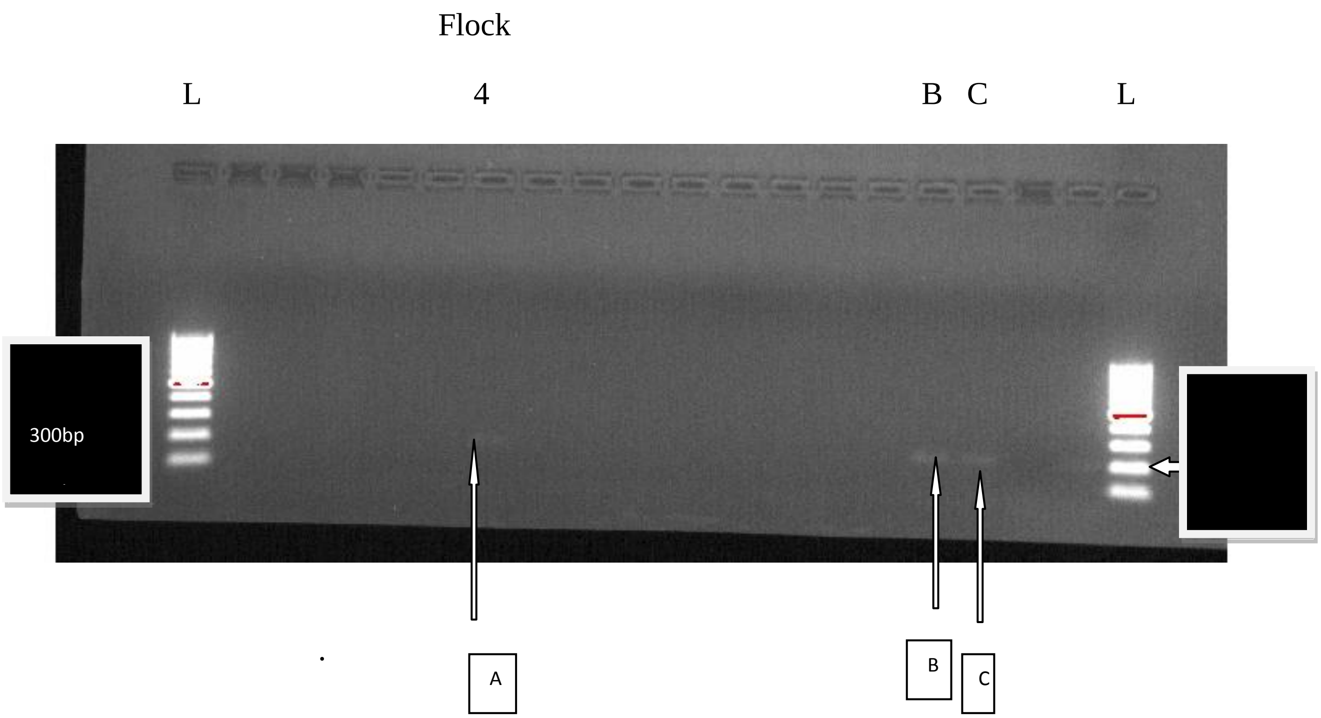

Plate III: Visualization of polymerase reaction product from flock 4 broiler chicken showing positive bursa (A) for chicken anaemia virus (B) and positive control (C). Horizontal arrow shows 186 bp which is specific for CIA antigen. Age of bird was 5 weeks. L (Ladder).

4.12 Age Distribution of Chickens with Chicken Infectious Anaemia Antibodies

Chickens aged 0-6 weeks were compared with respect to seropositivity with those 7-18 weeks (growers) and those that were above 19 weeks (adults) using odd ratio. The odds ratio analysis showed that chickens aged 7-18 weeks were 1.73 times more likely to have chicken infectious anemia antibodies than chickens 0-6 weeks of age. Also, chickens aged above 19 weeks were 7.29 times more likely to have antibodies to chicken infectious anaemia than those 0-6 weeks old. The Chi square (53.69)($p < 0.0001$) showed that there was association between age and seropositivity to chicken infectious antibodies and is significant. The 7-18 weeks chickens with odds ratio of 1.73 was not statistically significant at 95% confidence interval but the 19 weeks and above with odds ratio of 7.29 was statistically significant at the 95% confidence interval when both age groups were compared to 0-6 weeks chickens (Table 4.9).

4.13 Gross Lesions Observed in Flocks Diagnosed with Infectious Bursal Disease

4.13.1 Gross lesions observed in flock 22

At postmortem, turgid oedematous bursa of Fabricius, haemorrhages in the intestine and caeca tonsil were observed (Table 4.10).

4.13.2 Gross lesions observed in flock 43

At postmortem, haemorrhages in the proventriculus, intestine, caeca tonsils and haemorrhagic trachea were observed (Table 4.10).

4.13.3 Gross lesions observed in flock 45

At postmortem, haemorrhages in thigh muscles, enlarged turgid and haemorrhagic bursa of Fabricius, haemorrhages in the intestine, congestion of lungs and leg muscles were observed (Table 4.10).

4.13.4 Gross lesions observed in flock 50

At postmortem, turgid oedematous bursa of Fabricius without haemorrhages, hemorrhages in the proventriculus and intestine, congestion of muscles and trachea were observed in the flock (Table 4.10).

4.13.5 Gross lesions observed in flock 52

At postmortem, turgid oedematous bursa of Fabricius, haemorrhages in the proventriculus and intestine, and congestion of muscles were observed (Table 4.10).

4.13.6 Gross lesions observed in flock 57

At postmortem, haemorrhages in thigh muscles, enlarged turgid and haemorrhagic bursa of Fabricius, haemorrhages in the proventriculus, congestion of liver, spleen and muscles were observed (Table 4.10).

4.13.7 Gross lesions observed in flock 58

At postmortem, turgid oedematous bursa of Fabricius, haemorrhages in the proventriculus, intestine, caeca tonsils and trachea, congestion of liver and muscles were observed (Table 4.10).

4.13.8 Summary of gross lesions observed in flocks diagnosed with infectious bursal disease

Haemorrhages in thigh and pectoral muscles were observed by 28.6% (2/7) of the flocks, 28.6% (2/7) had enlarged, turgid and haemorrhagic bursa of Fabricius, 57.1% (4/7) had turgid, oedematous bursa of Fabricius, 71.4% (5/7) had haemorrhages in the proventriculus, 85.7% (6/7) had haemorrhages in the intestine, 42.9% (3/7) had haemorrhages in the caeca tonsils, 28.6% (2/7) had haemorrhagic trachea and 71.4% (5/7) had internal organs (liver, lungs, spleen) and muscles congested (Table 4.10).

4.13.9 Gross lesions of flock positive for chicken infectious anaemia virus antigens (flock 15)

At postmortem, congested muscles, distended gall bladder, haemorrhagic trachea, ballooned duodenum, ileum and jejunum, haemorrhagic caeca tonsils, enlarged liver and spleen, haemorrhagic and prominent proventricular glands and severely congested carcass were observed.

Table 4.9: Age distribution of chicken infectious anaemia antibodies in chickens from Zaria and environs, Nigeria between year 2013-2014.

	Chicken infectious anemia antibodies				
Age (weeks)	No. positive	No. negative	OR	95% CI	p-value
0-6	26	51	Ref		
7-18	51	58	1.73	0.94-3.16	0.076
≥ 19	130	35	7.29	3.99- 13.3	< 0.0001
Total	207	144			

Chi square = 53.69

P < 0.0001

<u>Key</u>

+ = positive for chicken anemia virus antibodies,

- = negative for chicken anemia virus antibodies

OR= odds ratio

CI = confidence interval

Table 4.10: Gross lesions observed in flocks diagnosed with infectious bursal disease using agar gel immunodiffusion test in Zaria and environs, Nigeria.

	Flocks							
Gross lesions	22	43	45	50	52	57	58	+ve (%)
Haemorrhages on thigh muscles/pectoral muscles	-	-	+	-	-	+	-	2/7 (28.6)
Turgid and haemorrhagic bursa of Fabricius	-	-	+	-	-	+	-	2/7 (28.6)
Turgid, oedematous bursa of Fabricius	+	-	-	+	+	-	+	4/7 (57.1)
Haemorrhages in the proventriculus	-	+	-	+	+	+	+	5/7 (71.4)
Haemorrhages in intestine	+	+	+	+	+	-	+	6/7 (85.7)
Haemorrhages in caeca tonsils	+	+	-	-	-	-	+	3/7 (42.9)
Haemorrhagic trachea	-	+	-	-	-	-	+	2/7 (28.6)
Congestion of liver, lungs, spleen, muscles	-	-	+	+	+	+	+	5/7 (71.4)

Key

+ = presence of gross lesion

- = absence of gross lesion

4.14 Prevalence of Infectious Bursal Disease Based on Type of Chicken and Association Between Type of Chicken and Infection with Infectious Bursal Disease Virus

Out of the 30 broilers tested for IBDV, 6 (20%) were positive for infectious bursal disease virus antigen, for the layers 25.64% (10/39) were positive for infectious bursal disease virus. Chi square showed that there was no association between type of chicken and infection with infectious bursal disease virus (chi square = 0.3029, $p = 0.582$) and was not statistically significant (Table 4.11).

4.15 Seroprevalence of Chicken Infectious Anaemia Based on Type of Chicken and Association Between Type of Chicken and Seropositivity

Out of 69 broilers, 46.51% (60/129) were seropositive to chicken infectious anaemia. Out of 93 breeders, 92.42% (86/93) were seropositive and out of 129 layers, 47.29% (61/129) were seropositive. The chi square ($X^2 = 58.7$, $P < 0.0001$, $\alpha < 0.05$) showed that there was association between type of chicken and seropositivity to chicken infectious anaemia antibodies which was significant at 95% confidence interval (Table 4.12).

4.16 Seroprevalence of Infectious Bursal Disease Based on Type of Chicken And Association Between Type of Chicken and Seropositivity

For layers, 26.17% (28/107) were seropositive to infectious bursal disease, for broilers 42.57% (63/148) were seropositive, for breeders 26.37% (24/91) were seropositive. Chi square showed that an association existed between type of chicken and seropositivity to infectious bursal disease and was statistically significant (Chi square = 10.15, $p = 0.0063$, $\alpha < 0.05$) (Table 4.13).

Table 4.11: Prevalence of infectious bursal disease in chickens presented to the Ahmadu Bello University Teaching Hospital Zaria, Nigeria between 2013-2014.

Type of chicken	No. of sample tested	No. positive (%)
Broiler	30	6 (20.00)
Layer	39	10 (25.64)
Total	69	16 (23.19)

Chi square = 0.3029 at 1 df

P value = 0.5820

OR for broiler= 0.725, 95% confidence interval= 0.2301 to 2.285

OR for layer = 1.379, 95% confidence interval= 0.4377 to 4.347

Table 4.12: Seroprevalence of chicken infectious anaemia based on type of chickens in Zaria and environs, Nigeria between year 2013-2014.

Type of chicken	No. of samples tested	No. positive (%)
Broiler	149	60 (40.26)
Breeder	93	86 (92.47)
Layer	109	61 (55.96)
Total	351	207 (58.97)

Chi square= 65.09

P< 0.0001

*OR for broiler = 0.2522, 95% confidence interval= 0.1607 to 0.3959

*OR for breeder = 13.91, 95% confidence interval =6.198 to 31.22

OR for layer = 0.8356, 95% confidence interval = 0.5288 to 1.321

*Significant at P< 0.05

Table 4.13: Seroprevalence of infectious bursal disease based on type of chickens in Zaria and environs, Nigeria between year 2013-2014.

Type of chicken	No. of samples tested	No. positive (%)
Layer	107	28 (26.17)
Broiler	148	63 (42.57)
Breeder	91	24 (26.37)
Total	346	115 (33.24)

$X^2 = 10.15$

$P = 0.0063$

OR for layer = 0.6192, 95% confidence interval= 0.3736 to 1.026

*OR for broiler = 2.081, 95% confidence interval = 1.321 to 3.278

OR breeder = 0.6456, 95% confidence interval = 0.3792 to 1.099

*Significant at $P < 0.05$

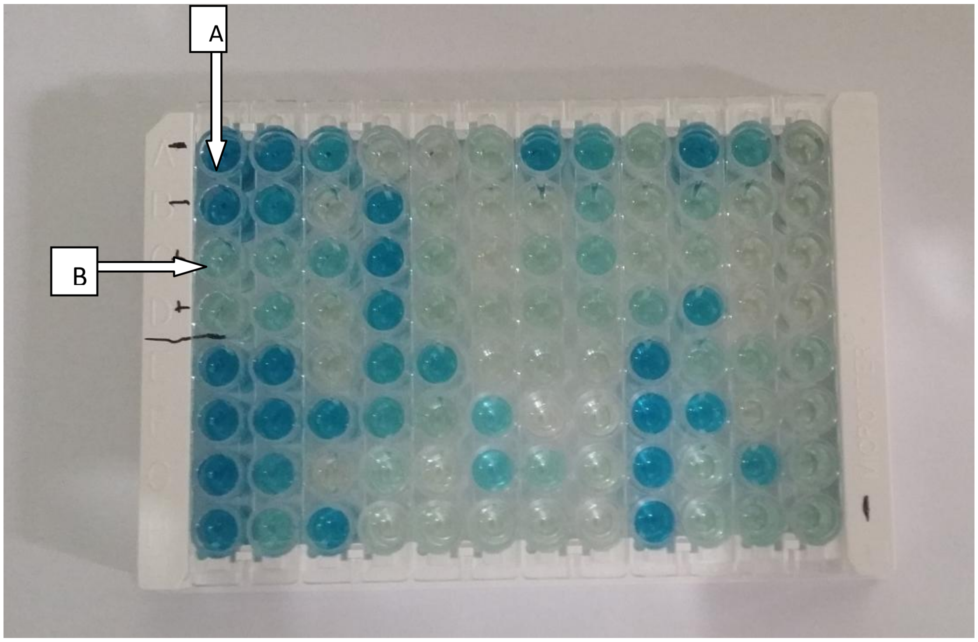

Plate IV: Enzyme linked immunosorbent assay for chicken infectious anaemia showing negative (A) and positive (B) colour of antigen-antibody reaction for sera tested.

Absorbance for A = greater than or equal to 0.6   B = less than 0.5

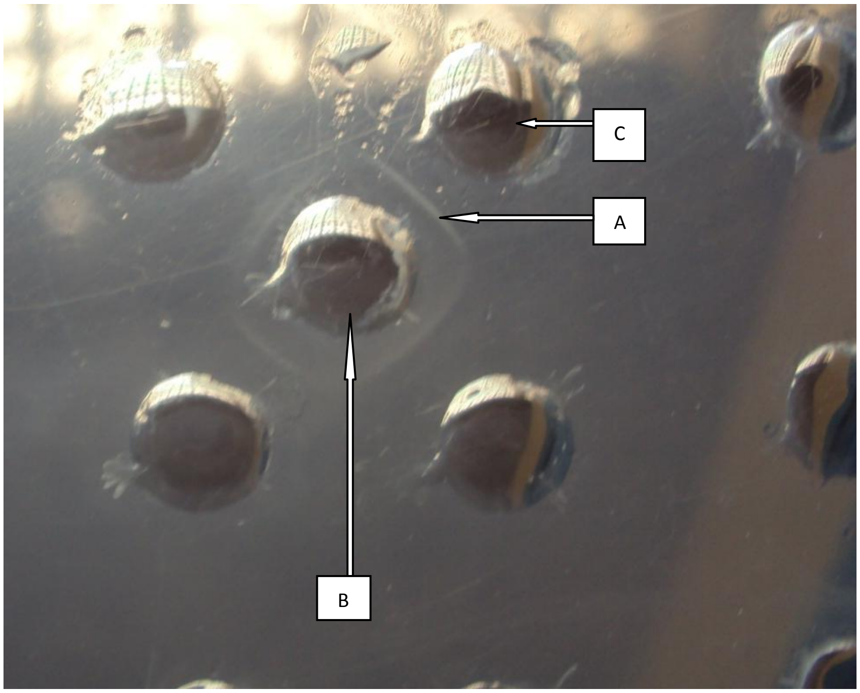

Plate V: Agar gel immunodiffusion test showing positive wells with a precipitin line A between central well B (IBD antigen) and peripheral well C (positive well for IBD antibodies).

CHAPTER FIVE

DISCUSSION

In this study, CIAV and IBDV were shown to be present in Zaria, Nigeria based on the polymerase chain reaction and agar gel immunodiffusion, respectively. Infectious bursal disease is commonly diagnosed in Zaria, but CIA is rarely suspected. Therefore, the detection of CIA viruses in Zaria is novel and of clinical importance.

The clinical signs observed in some flocks that tested positive to CIA antibodies in this study which were paleness, unwillingness to move, drooped wings, ruffled feathers, uneven growth and weight loss were similar to those observed by Todd *et al.* (2001). However, in this study depression and anorexia were not observed. Unwillingness to move occurred more frequently and may be attributable to anaemia, weakness and secondary bacterial infection.

Of all the flocks sampled, 57.4% were positive for CIA antibodies, with an overall seroprevalence of 58.97 % and flock seroprevalence ranging from 12.5 % - 100%. These values are close to those obtained by Owoade *et al.* (2004) in Ibadan, South West Nigeria with an overall prevalence of 55.7 % and overall flock prevalence of 55%, with individual flock seroprevalence which ranged from 16.6 % - 100 %. It is likely that chicken infectious anaemia virus has similar epidemiological pattern in every area it is found.

A total of 48.9% (23/47) of the flocks tested were seropositive for IBD antibodies. When compared to the value for CIA above, it is obvious that more flocks were exposed to CIA virus than IBD virus in Zaria and environs. The seroprevalence of IBD may be said to have been exaggerated because of inclusion of IBD vaccination in the programme for Zaria assuming that agar gel immunodiffusion does not discriminate antibody response to IBD vaccine from field virus. As vaccination of breeders against CIA is not practiced in Nigeria as noted by Owoade *et al*. (2004), confirming that the high seroprevalence in Nigeria is due to infection by chicken anemia virus and not antibody response to CIA vaccine.

In flock 45 and 57, the observation made that in the same birds, IBDV can only be detected if IBD antibodies are absent may be explained by the competitive blocking enzyme linked immunosorbent assay principle in the sense that viruses that have already been bound by the primary antibodies will not be able to be bound by the secondary antibodies (OIE, 2008). This assumption can only be true if serology was the test used to detect antigens and antibodies. However, more sensitive test like polymerase chain reaction may be able to detect the IBD antigens even if viral antigens or viruses have just formed antigen- antibody complexes.

Birds aged 19 weeks old and above were 7.29 times more seropositive when compared to chicks 6 weeks old and less. Growers (7-18 weeks old) were 1.73 times more seropositive when compared to chicks (6 weeks old and less). From odd ratios older birds appeared to have developed either protective antibodies or antibodies resulting from field exposure. But the serological assay used to detect the antibodies specified that at the dilution of the sera as recommended, one will be testing for field exposure to CIA virus. Based on this information it can be said that older birds have a higher chance of being seropositive after being infected horizontally than younger birds. This is supported by the finding of Snoeck *et al.* (2012) who reported that most seropositive flocks became seropositive after week five. The resulting odds ratio from the comparism of chickens of 7-18 weeks of age to chicks of 6 weeks and less which is 1.73 with a 95% confidence interval ranging from 0.94 to 3.16 has a Z-statistic of 1.753 and an associated p- value which equals 0.083. For this p-value, the assumption is that although there was a 1.73- fold increased odds of CIA antibody positive chickens in 9-20 weeks chickens compared to chicks 6 weeks and less, this increase is not statistically significant at the 5% level. Chickens of 7-18 weeks may have had more seropositives than chicks because they would possibly be seroconverting. This claim can be supported by the finding of Snoeck *et al*. (2012) that most seropositive flocks became seropositive after week five.

The resulting odds ratio from the comparison of CIA seropositive chickens of above 19 weeks old to chicks of 6 weeks and less which is 7.29 with a 95% confidence interval ranging from 3.99 to 13.3 has a Z-statistic of 1.753 and an associated p-value which less than 0.05. For this p-value the assumption is that there is a 7.29- fold increased odds of CIA antibody positive chickens in > 19 weeks chickens compared to chicks 6 weeks and less, and this increase is statistically significant at the 5% level. The high number of seropositives in this age group may likely be due to completion of seroconversion of most CIA virus infected flocks.

The odds of detecting antibodies to CIA when antibodies to IBD is present in a flock is 2.89 with a 95% confidence interval ranging from 2.121 to 3.932. The Z- statistic is 6.758 and the associated P value less than 0.05. The assumption is that there is a 2.89 fold increase odds of finding CIA antibody positives compared to IBD antibody positives, and the increase is statistically significant at the 5% level.

The gross lesions observed for flocks 22, 43, 45, 50, 52, 57 and 58 were similar to those described for IBD which were turgid, oedematous, and sometimes haemorrhagic bursa of Fabricius (Tsukamoto *et al*., 1992). Van den Berg (2000) also observed ecchymotic haemorrhages in the muscles and mucosa of the proventriculus in majority of birds affected by IBD. This shows that the agar gel immunodiffusion test when used to diagnose IBD by detecting IBDV antigens is actually very sensitive as positive cases showed lesions consistent with those in the literature (OIE, 2008). However, haemorrhages were found in the intestines, caeca tonsils and trachea in some flocks. Of the accepted gross lesions, inflammation of the bursa of Fabricius pointed more to IBD. Though, haemorrhages of the above mentioned organs; bursa of Fabricius, muscles and proventriculus was characteristic of IBD in all the flocks that comfirmatory diagnoses was made by agar gel immunodiffusion test for detection of IBD viral antigen. The only gross lesion that points to CIA was the haemorrhagic and prominent proventricular glands which could be mistaken for IBD.

The flock prevalence obtained by testing thymus from 26 flocks for CIA antigen with polymerase chain reaction was 3.8%. This low overall prevalence for broilers and layers may be due to maternally derived immunity of offsprings of breeders to CIA virus, since most breeders would have seroconverted before point of lay. Most offsprings will therefore be protected from CIA. However, this research confirms the presence of CIAV in an exotic backyard flock other than a breeder flock in Zaria, Kaduna State. The location of the farm was at Gyellesu. The flock prevalence of 3.6% obtained by testing the bursa was close to that of the thymus which was 3.8%.

Co-infection with both CIA and IBD viruses may be said to have occurred in the early age of the birds that tested positive for both CIA and IBD antibodies if the antibodies detected were as a result of immunological response to the viruses considering that maternally derived antibodies of CIA disappear at about 3 weeks of age and maternally derived antibodies to IBD wanes off at about 2 weeks of age (McNulty *et al*., 1988; McNulty, 1991). It is important to note also that chickens are most susceptible to IBD between 3 and 6 weeks of age which coincide with that of CIA as stated by Engstrom and Luthman (1984) and Yuasa *et al*. (1987) that the first signs of CIA usually occur towards the end of the second week of life. The CIA is acute with peak mortality that occurs within 5 to 6 days of onset of disease signs and mortality often declines to normal level after a further 5 to 6 days. Although, it is worthy to note that IBD can occur in chicks less than 3 weeks of life when they do not have maternally derived antibodies. In this study, 38.3% of the flocks had both CIA antibodies and IBD antibodies. A more conclusive result would rather be by detecting antigens of both viruses in the same chickens because some of our results may be infections at different ages. In terms of association between CIA and IBD, the chi-square test showed that an association exists in this study. This is in agreement with the work of

Hagood *et al*. (2000) who stated that a highly significant association has been demonstrated between CIA and other diseases such as coccidiosis, gangrenous dermatitis or respiratory disease.

Actual coinfection of CIAV and IBDV investigated by looking for their antigens was detected in only one flock. The probability of having coinfection of both CIAV and IBDV was possible but it was very slim. This may be due to the small size of flocks tested. This finding is in agreement with previous work of Owoade *et al*. (2010) in which co-infections were found.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

From this work it was concluded that:

1. Chicken infectious anaemia virus (3.17% of thymus and 1.49% of bursae) was present in Zaria while infectious bursal disease virus (23.2% from bursae) was detected.
2. Co-infection of CIAV and IBDV (3.4%) was detected in broiler chickens of 7 weeks of age.
3. Chicken infectious anaemia antibodies were 2.89 times more likely to be present in birds when IBD antibodies are detected.
4. Gross lesions observed for chickens that tested positive for CIA were: congested muscles, distended gall bladder, haemorrhagic trachea, ballooned duodenum, ileum and jejunum, haemorrhagic caeca tonsils, enlarged liver and spleen, haemorrhagic and prominent proventricular glands and severely congested carcass.
5. The seroprevalence of infectious bursal disease was 33.2% while that of CIA was 58.97%.
6. Antibodies to CIA and IBD can be detected in the same flock (38.3%).
7. Adult chickens (greater than 19 weeks old) were 7.29 times most likely to be seropositive to CIA when compared to chicks (less or equal to 6 weeks old).

6.2 Recommendations

From this study it was recommended that:

1. Further study should increase the sample size of the thymus and bursae in order to establish more accurately the probability of co-infection of CIAV and IBDV in Zaria.
2. Further research should be carried out to characterize the CIA virus detected in Zaria.
3. Improved biosecurity practices is recommended on poultry farms in other to prevent infection by CIAV and IBDV.
4. It is recommended that breeders be vaccinated against chicken infectious anaemia to prevent transmitting CIAV to their offspring. In addition vaccinated breeders would develop protective antibodies that will be transmitted to their offspring.
5. CIA should be included as a differential diagnosis to IBD whenever clinicians are making diagnosis in Zaria.

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APPENDICES

Appendix I: Department of Veterinary Medicine, Ahmadu Bello University, Zaria, Avian Clinic Record Sheet.

Information about farm/ flock

Date:

Case No.:

S/No:

Owners name:

Address:

Source of birds:

Flock size:

Age of birds:

Specie/ Breed/Type:

Source of feed:

Vaccinations (Types/Date) :

Date of onset of disease:

Number sick / morbidity:

Number dead and mortality pattern:

Number presented for Examination:

Clinical signs noticed:

Gross lesions:

Samples to be collected from each bird:

1. Blood sample
2. Bursa of Fabricius
3. Thymus

Diagnosis made:

Appendix II: Sources and types of samples collected for laboratory test for chicken infectious anaemia and infectious bursal disease antibodies and antigens in Zaria and environs.

	Type of sample		
Source of sample	No. of blood collected(%)	No. of bursa collected(%)	No. of thymus collected(%)
Ahmadu Bello University Veterinary Teaching Hospital	248 (70.7)	55 (79.7)	55 (87.3)
Private clinic Palladan	6 (1.7)	14 (20.3)	8 (12.7)
Samaru live bird market	65 (18.5)	0 (0)	0 (0)
Sabon gari live bird market	32 (9.1)	0 (0)	0 (0)
Total	351 (100)	69 (100)	63 (100)

Appendix III: Composition of reagents used in enzyme linked immunosorbent assay.

Reagents	Constituents
Antigen	CIAV antigen coated plates preserved with kathon
Positive control	Diluted chicken anti-CIAV serum preserved with sodium azide
Negative control	Diluted chicken serum non-reactive to CIAV preserved with sodium azide
Conjugate	anti-CIAV: horse radish peroxidase conjugate preserved with gentamicin and kathon
Sample diluent	Preserved with sodium azide
TMB substrate	3,3’,5,5’- tetramethylbenzidine
Stop solution	Sodium hydroxide
Wash concentrate (10x)	Phosphate buffered saline, tween 20