REPRODUCTIVE ENDOCRINE AND CLINICO-PATHOLOGICAL CHANGES INDUCED BY *Escherichia coli* EXPERIMENTAL INFECTION IN LAYERS

BY

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DECLARATION

I declare that the work in this Dissertation entitled "Reproductive endocrine and clinico-pathological changes induced by *Escherichia coli* experimental infection in layers" has been performed by me in the Department of Veterinary Pathology, 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.

Joshua Tersue ADEKE	_____	_____
NAME OF STUDENT	SIGNATURE	DATE

CERTIFICATION

This dissertation entitled **"REPRODUCTIVE ENDOCRINE AND CLINICO-PATHOLOGICAL CHANGES INDUCED BY *Escherichia coli* EXPERIMENTAL INFECTION IN LAYERS"** by Joshua Tersue ADEKE meets the regulations governing the award of the degree of Master of Science in Veterinary Pathology of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

PROF. K. A. N. ESIEVO Chairman, Supervisory Committee	_____ Signature	_____ Date
PROF. SANI ADAMU Member, Supervisory Committee	_____ Signature	_____ Date
PROF. S. B. OLADELE Head of Department	_____ Signature	_____ Date
PROF. S.Z. ABUBAKAR Dean, School of Postgraduate Studies	_____ Signature	_____ Date

DEDICATION

This dissertation is dedicated to my parents (Mr and Mrs Andrew Iorkyaa Adeke) and to my nine siblings (Abraham, Abigail, Abel, Comfort, Veronica, Lydia, Deborah, Ruth, and Ephraim) for their love and prayers.

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ABSTRACT

This study determined the reproductive endocrine and clinico-pathological changes associated with *Escherchia coli* (*E. coli*) infection in layer chickens. A total of 20 laying chickens (20 weeks old) were acquired and assigned, at random, into two groups (infected and control) of 10 layers each. Each of the birds in the infected group was challenged with 0.5 ml of bacterial aliquot containing 10^9 colony forming units (CFU) of the bacteria, administered intratracheally. Three (3) ml of blood were collected from each bird in both groups and used for haematological and biochemical analyses. *E. coli* infection in the layers caused reduction in feed and water consumption, watery yellowish faeces and weakness by day 3 post infection (pi) and also decreased mean oestrogen profile from day 6 (347 ± 2.55 pg/ml) reached the lowest value (332.6 ± 5.41 pg/ml) on day 28 pi that differed significantly ($p < 0.05$) from the corresponding value in the control group. The mean plasma progesterone profile in the infected group started from day 4 pi and reached the lowest significant ($p<0.05$) value (94.8 ± 1.98 pg/ml) on day 28 pi, against the control group. The mean plasma calcium concentration decreased from day 6 to reach a lowest level (1.61 ± 0.11 mmol/L) on day 14 pi, which was significantly different ($p < 0.05$) from that in the control group. The mean plasma phosphate concentration increased from day 6 to a significantly ($p < 0.05$) higher level (1.64 ± 0.12 mmol/L) on day 14. The mean parathormone profile increased from day 6 (24.10 ± 0.56 pg/ml) to a significantly ($p < 0.05$) higher level on day 21 (31.04 ± 0.80 pg/ml). The mean plasma aspartate aminotransferase (AST) activity in the infected group progressively increased from day 6 pi to reach a significantly higher ($p < 0.05$) value (66.4 ± 0.93 IU/L) on day 28 pi while the mean plasma alanine aminotransferase (ALT) activity progressively increased in the infected group beginning from day 6 pi to attain the highest significant ($p < 0.05$) value (74.4 ± 2.11 IU/L) on day 21 pi compared to the control group. The mean plasma total protein concentration in the infected group decreased from day 4 pi

and reached the lowest significant ($p < 0.05$) value (5.12 ± 0.28 mg/dl) on day 35 pi when compared with the control. The mean PCV decreased significantly ($p < 0.05$) in the infected group to the lowest mean value (17 ± 0.71%) on day 4 and then increased to a peak level (24.6 ± 1.03%) on day 28. The mean total white blood cell count increased significantly ($p < 0.05$) in the infected group from day 2 (14.81 ± 3.28 x 10^9/L) to a peak level (29.16 ± 0.81 x 10^9/L) on day 14 pi. Post-mortem examination grossly revealed congested lungs, enlarged liver and kidney, hyperaemic intestine, congested ovarian follicles and yellowish fibrinous material in the infected group. Histopathological findings caused by the infection were congested ovarian blood vessels, mononuclear cellular infiltration and generalised necrosis of the liver, kidney and heart. In conclusion, the *E. coli*-induced reproductive endocrine and clinicopathological changes recorded in this experimental study may partly be responsible for the reported reproductive disorders in layers in layers infected with *E. coli*.

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LIST OF ABBREVIATIONS, DEFINITIONS AND SYMBOLS USED

Ad libitum - Allowed unrestricted access

ALT - Alanine aminotransferase

APEC - Avian pathogenic *E. coli*

AST - Aspartate aminotransferase

Ca - Calcium

CFU - Colony forming units

E. coli - *Escherichia coli*

EDTA - Ethylene diamine tetra acetic acid

ELISA - Enzyme linked immunosorbent assay

EMB - Eosin and methyline blue

et al - And others

FAO - Food and Agricultural Organization

fl - Femtolitre

g - Gram

g/dl - Gram per deciliter

g/dl - Grams per decilitre

H & E - Hematoxyline and eosin

Hb - Haemoglobin

LH - Luteinizing hormone

mg/dl - Milligram per decilitre

mmol - Million mole

mm - Millimetre

ml - Millilitre

P4 - Progesterone

RBC - Red blood cell

TMB - Tetramethylbenzidine

TP - Total protein

TWBC - Total white blood cell

WBC - White blood cell

LPS - Lipopolysaccharide

IU - International unit

Ltd - Limited

MCV - Mean corpuscular volume

MCHC - Mean corpuscular heamoglobin concentration

PCV - Packed cell volume

pg/dl - Pictogram per deciliter

P - Phosphorus

PTH - Parathyroid hormone

Pi - Post-infection

spp - Species

TP - Total protein

U/L - Units per litre

^{0}C - Degree centigrade (degree Celcius)

µl - Microlitre

% - Percent

& - And

UK - United Kingdom

USA - United State of America

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of the study

Colibacillosis, a disease caused by *Escherichia coli (E. coli),* is one of the most common infectious diseases in the poultry industry. Most *E. coli* serotypes are non-pathogenic and found forming part of microflora of the gastro-intestinal tracts of birds, reptiles and mammals. About 10-15% of the intestinal coliforms belong to pathogenic serotypes (Wray and Davies, 2001; Rahman *et al*., 2004). Therefore, birds are continuously exposed to infection with these organisms through faecal contamination of feeds, water and the environment (Charlton, 2006). Vertical transmission of the disease occasionally occurred (Lutful-Kabir, 2010). However, Avian Pathogenic *Escherichia coli* (APEC) serotypes, as a group, are virulent for birds in which they cause the disease, avian colibacillosis. All types and age groups of birds are susceptible to the disease, ranging from chicks to adult layers, broilers and breeders. These APEC infections were reported to be the main cause of morbidity, depressed feed conversion efficiency, decreased growth rate, low productivity and high mortality rates in affected poultry flocks (Dho-Moulin and Fairbrother, 1999; Alterkruse *et al.*, 2002; Barnes *et al*., 2003; Ewers *et al*., 2003; Barnes *et al.*, 2008). The infections were traditionally associated with losses in broilers, but in Europe from the mid-nineties on, they had been increasingly observed in layer hens (Zanella *et al.*, 2000; Vandekerchove *et al.*, 2004a).

Whereas colibacillosis is primarily an enteric disease in mammals, in poultry it may be a localized or systemic disease, occurring mostly secondary to impairment of the host defense mechanisms (Saif, 2003). The acute form of the disease was characterized by septicaemia, which results in death, while its subacute form is also accompanied by pericarditis,

airsacculitis and perihepatitis, reproductive tract infection like salpingitis and/or egg peritonitis, resulting in huge mortality (Landman and Cornelissen, 2006; Ozaki and Murase, 2009). *Escherichia coli* infections had also been reported in turkeys, geese, and ducks, in which they caused significant economic losses (Landman and Cornelissen, 2006).

1.2 Statement of the Research Problem

Avian Pathogenic *Escherichia coli* (APEC)-infected layers were reported to suffer from reproductive disorders, which include oophoritis, salpingitis, with consequent effects on egg production (Jordan *et al.,* 2005; Timothy *et al.,* 2008). Though, oophoritis and/or salphingitis were identified as part of the reported reproductive disorders in APEC-infected layers, there is dearth of information in available literature on the effect of the infection with this organism on reproductive endocrine function in layers. Progesterone in birds plays an important role in the regulation of several functions such as ovulation, gonad differentiation, and sexual and nesting behaviours (Johnson *et al.*, 1985; Appleby, 1986; Nitta *et al.*, 1991; Yoshimura and Bahr, 1991; Camacho-arroyo *et al.*, 2006). On the other hand, oestrogens promote the formation of the vitellogenins from the liver, which are lipoproteins that are incorporated into egg yolk. These lipoproteins bind calcium, and their production is followed by a rise in serum calcium levels. This oestrogen-controlled hypercalcemic effect is not seen in mammals and is thought to be due to the need to produce large calcified eggs, requiring a rapidly mobilized source of calcium (Dacke, 2000). It is speculated that avian colibacillosis may disrupt the synthesis and release of these hormones, which could further aggravate reproductive disorders in the layers.

Also, colisepticaemia (Ewers *et al*., 2003 Vandekerchove *et al*., 2004b; Singh *et al.*, 2011), oophoritis (Lutful-Kabir, 2010), salphingitis (Bisgaard and Dam, 1980), airsacculitis (Harry, 1964), perihepatitis, as reported lesions in layers with colibacillosis, could have effect on

their haematological parameters. It is pertinent to determine the reproductive endocrine and other haemato-biochemical changes that occur and lead to the reported reproductive disorders in layers with colibacillosis.

1.3 Justification of the Study

Poultry production is a big industry in Nigeria and is an important component of the livestock sub-sector that provides employment, income, animal protein for urban and rural dwellers as well as manure for crop production (Kekocha, 1994; Laseinde, 1994; Folorunsho and Onibi, 2005; Obi *et al.,* 2008).

Avian colibacillosis is considered as one of the major bacterial diseases that afflicts the poultry industry worldwide, and along with salmonellosis, is also one of the most common avian diseases communicable to humans (Gross, 1994; Barnes *et al.*, 2003, 2008; Lutful-Kabir, 2010; Singh *et al.,* 2011). Despite the recognition of the disease for over a century, avian colibacillosis remained majorly endemic with consequent huge economic losses (Otaki, 1995; Dho-Moulin and Fairbrother, 1999).

The search for laboratory diagnosis of the reproductive disorders reported in layers with colibacillosis requires establishing the specific endocrine and other haemato-biochemical alterations, consequent to the disease, which necessitated this study.

1.4 Aim of the Study

The aim of this study was to determine the reproductive endocrine and clinico-pathological changes in layers experimentally infected with *E. coli*.

1.5 Objectives of the Study

The objectives of the study were to determine the changes in the:

i. Plasma progesterone, oestrogen and parathormone profiles of layers experimentally infected with *E. coli*.

ii. Plasma calcium and phosphorus concentrations of layers experimentally infected with *E. coli*.

iii. Haematological profile of layers experimentally infected with *E. coli.*

iv. Plasma activities of markers of hepatic dysfunction in layers experimentally infected with *E. coli*.

v. Gross and microscopic features in some selected organs of layers experimentally infected with *E. coli*.

1.6 Research Questions

i. Does experimental infection with *E. coli* cause alterations in plasma profiles of progesterone, oestrogen and parathormone in layers?

ii. Does experimental infection with *E. coli* cause alterations in plasma calcium and phosphorus concentrations in layers?

iii. Does experimental infection with *E. coli* have any effect on haematological parameters of layers?

iv. Does experimental infection with *E. coli* cause alterations in plasma concentrations of markers of hepatic dysfunction in layers?

v. Does experimental infection with *E. coli* cause gross and histopathological changes in some selected organs of layers?

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Avian Colibacillosis

Colibacillosis, caused by avian pathogenic *Escherichia coli* (APEC) infections in chickens and other avian species is responsible for significant economic losses, particularly, in the poultry industry, worldwide (Barnes *et al.,* 2008). It causes high morbidity and mortality in broiler chickens and laying hens (Barbieri *et al*., 2015). The losses are related to the condemnation of carcasses in slaughterhouses, mortality, and severe decreases in egg production (Dho-Moulin and Fairbrother, 1999; Barnes *et al*., 2008). In particular, APEC-induced peritonitis–salpingitis in commercial layer chickens is a serious concern to the egg industry (Barnes *et al.,* 2008; Vandekerchove, 2004a; Zanella, 2000). Peritonitis is usually restricted to laying hens in production and is considered as one form of colibacillosis, which may be accompanied by lesions such as perihepatitis, septicemia, and salpingitis (Jordan, *et al.,* 2005; Lindgren, 1963; Vandekerchove, 2004b; Zanella, 2000). It has been reported that when peritonitis occurs alone, it is most likely to be an acute disease and the infection may more likely be introduced via the air sacs, whereas when it occurs together with salpingitis and salpingoperitonitis (SPS) the infection may more likely be introduced via the oviduct (Jordan *et al*., 2005). Thus, the APEC-induced salpingitis, peritonitis, and SPS in domestic hens are collectively referred to as egg peritonitis (Jordan *et al*., 2005). The oviducts containing APEC remain infected for a long period (Gross, 1958; Trampel, 2007) and serve as carriers for the infection (Ardrey, 1968), which could further ascend into the peritoneum and progress into peritonitis. Egg peritonitis induces acute mortality and is characterized by the appearance of a localized yellow exudate deposition on serosal surfaces that may be disseminated throughout the body cavity and result in carcass condemnation (Lindgren, 1964; Zanella *et al.,* 2000).

2.2 Aetiologic Agent of Avian Colibacillosis

Avian pathogenic *E. coli* (APEC) is the etiologic agent of extra-intestinal infections in broiler chickens and laying hens, and these are collectively known as colibacillosis (Barbieri *et al.*, 2015). An *E. coli* strain can be designated as APEC when isolated from birds with characteristics of colibacillosis lesions and birds that were killed by this bacterium. *E. coli* designated as APEC must possess some virulence genes such as encoding adhesins, iron-scavenging systems, protectins, and other virulence traits (Babai *et al*., 2000; Johnson *et al*., 2008; Ewers *et al*., 2009).

2.2.1 The virulence factors of the bacterium

Several investigations have added to the knowledge about the pathogenic mechanisms expressed by APEC strains (Dho-Moulin and Fairbrother 1999). The virulence factors that have been described to be expressed by these strains include adhesins, toxins, iron uptake systems, and resistance to the host serum.

2.2.1.1 Adhesins

The bacterial adhesion to epithelial tissues is considered to be an important step for the establishment of the *E. coli* infection since it permits the bacterial linkage and maintenance in close contact to the host epithelial tissues (Moon, 1990).

Fimbriae are long hair like extracellular appendages that mediate specific attachment to the host epithelial cell surface. Binding is also mediated by non- fimbrial adhesins (Jones *et al.,* 1992, Antao *et al.*, 2009). A wide spectrum of fimbriae are expressed by extra-intestinal pathogenic *E. coli* (ExPEC) having different receptor specificities (Soto and Hultgren, 1999; Klemm and Schembri, 2000) providing the bacteria the capacity to bind to different target molecules. Fimbrial adhesins recognize specific molecular motifs enabling the bacterium to target specific surfaces, such as a specific tissue in gut or bladder of human and animal hosts or respiratory

tract. This phenomenon of tissue specificity is referred to as tissue tropism that relies on specific interaction with receptor targets and specific tissue surfaces (Klemm *et al.,* 2010).

There are many types of fimbriae in the APEC strains. Type I Fimbriae are related to the adhesive property of the organism to the avian upper respiratory tract (Wooley *et al*., 1998). The adhesive properties of Type 1 fimbriae are inhibited by specific anti-serum and by D-mannose, a carbohydrate that has its cellular receptor on the eukaryotic cell membrane. These characteristics are used for its characterization (Gyimah and Panigraphy, 1988). Pourbaskhsh *et al.* (1997) suggested that while Type 1 fimbriae are associated with the upper respiratory tract initial colonization, P fimbrial adhesin may be involved in the bacterial establishment in deeper avian organs. Wooley *et al.* (1998) also suggested that while Type 1 is necessary to initial colonization of the respiratory epithelium, additional factors like motility and colicin V production would be responsible by the persistence of the colonization and by the observed tracheal lesions development. P fimbriae were first described among *E. coli* strains associated with human urinary tract infections (UTI) (Kallenius *et al.,* 1980) being also found among APEC strains (Achtman *et al.,* 1983; Dozois *et al.,* 1992). P fimbriae are encoded by the *pap* operon that is located in the bacterial chromosome (Latham and Stamm, 1994). The role of P fimbriae in the APEC pathogenicity has not been completely elucidated yet. Pourbakhsh *et al.* (1997) using *in vivo* studies, verified that P fimbriae presented phase variation and suggested that these adhesins would not be important for the initial colonization of the upper respiratory tract, but it would be in the latter infection stages. Curli fimbriae are thin and curly appendages found on the cell surface of *Salmonella enterica* and *E. coli* (Olsén *et al*., 1989), and are responsible for the bacterial linkage to proteins of the extracellular matrix (Collinson *et al.,* 1993) and for bacterial survival in the external environment (Olsén *et al.,* 1993). Curli fimbriae are optimally expressed at 26 ºC during the growth in the stationary

phase and in low osmolarity medium (Olsén *et al.*, 1993). The genes responsible for curli fimbriae expression are encoded by two operons: *csg*BAC and *csg*DEFG (La Ragione and Woodward, 2002). Curli related sequences have been widely found among APEC strains. Maurer *et al.* (1998) detected *csg*A gene in all APEC strains analysed.

2.2.1.2 Iron acquisition systems

Most APEC strains survive and grow in environments with low iron availability, mainly inside the host, because the expression of iron acquisition systems (Dho and Lafont, 1984). The bacterial iron acquisition mechanisms include the production of siderophores that act as ion chelants in the host (Williams and Griffiths, 1992). Two types of siderophores are known: fenolates and hidroxamate. Aerobactin is a hidroxamate siderophore that is encoded by a plasmid operon (Gibson and Magrath, 1969; Williams, 1979). This siderophore is also found among fungi, enteroinvasive *E. coli* and APEC strains (Dho and Lafont, 1984; Waters and Crosa, 1991). Dho and Lafont (1984) observed a positive correlation between the low iron concentration, APEC growth ability and the lethality capacity to one-day old chickens observed in these strains.

2.2.1.3 Colicins

These are proteins expressed by *E. coli* that inhibit the bacterial growth from the same or related species. Colicins are compounded by two subunits: one that provokes bacterial cell lesions and the other that protects the bacterium against their own colicins (Hardy *et al.*, 1975). The majority of APEC strains have colicin V plasmids (Wray and Woodward, 1997).

2.2.1.4 Capsule

Some *E. coli* strains have an N-acetyl muramic acid capsule on their cell surface that interacts with the classical complement pathway conferring immune resistance to the bacteria and that

induces the immune resistance (Jann and Jann, 1977). K1 capsular antigen is frequently associated to APEC strains belonging to serogroups O1, O2 and to non typable strains (Gross, 1991). Pourbakhsh *et al.* (1997) demonstrated the three APEC strains expressing capsule K1 were more resistant to the serum bactericidal effects than APEC strains that expressed other K antigens.

2.2.1.5 Serum resistance

The bacterial resistance to the complement, mediated by bacterial surface structures like Lipopolysaccharide (LPS), capsule, Col V colicin, and outer membrane proteins, have been associated with APEC strains (Gross, 1991; Fantinatti *et al.*, 1994; Ngeleka *et al.*, 1996; Lynne *et al.*, 2007). Pfaff-McDonough *et al.* (2000) suggested that the increased serum survival (Iss) factor is associated with APEC pathogenicity since the *iss* gene have been found more frequently among pathogenic than non pathogenic strains, despite of serotype, avian species and lesion origin.

2.2.1.6 Toxins

Some APEC strains are able to produce toxins like labile temperature (LT) and stable temperature (ST) enterotoxins (Smith and Gyles 1969), and verotoxins known as Shiga-toxins (Stx) (O'Brien *et al*., 1977; 1982; Emery *et al.*, 1992; Parreira and Yano, 1998; Fantinatti *et al.*, 1994; Blanco *et al.*, 1997). APEC strains cytotoxic activity to Vero cells was observed by Fantinatti *et al.* (1994) and, Parreira and Yano (1998). Parreira and Gyles (2002), identified a Stx- gene among *E. coli* strains isolated from chickens suffering from cellulitis, septicemia, and swollen head syndrome and from sick turkeys. These same authors (Parreira and Gyles, 2003) described a vacuolating toxin to be expressed by an APEC strain. This toxin is encoded by the *vat* gene that belongs to a pathogenicity island and was later found to be expressed by another APEC strains (Ewers *et al.*, 2004; 2005).

2.3 Incubation Period

The time between infection and onset of clinical signs varies with the specific type of disease produced by *E. coli.* The incubation period is short, generally between 1 and 3 days, in experimental studies in which birds are exposed to high numbers of virulent organisms. In the field it is more common to see colisepticemia 5 – 7 days after infection with a predisposing agent such as infectious bronchitis virus in chickens or haemorrhagic enteritis virus in turkeys (Barnes *et al.,* 2008).

2.4 Clinical Signs

Clinical signs of collibacillosis vary from inapparent to total unresponsiveness just prior to death depending on the specific type of disease produced by *E. coli.* Localized infections generally result in fewer and milder clinical signs than systemic diseases. Birds with colisepticemia are often terminally moribund and the flock may be inactive and not eating. Decreased water consumption is associated with a poor prognosis (Barnes *et al.,* 2008). Severely affected individual birds are unresponsive when approached, do not react to stimuli, and are easily caught and handled. They sit with their eyes closed in a hunched position with drooping of the head, neck, and wings. The beak may be inserted into the litter to support the head. Dehydration is indicated by dark dry skin, which is especially noticeable in the shanks and feet. Dehydrated young chicks typically have prominent raised folds of skin along the medial and lateral sides of the shanks and toenails that appear black. Although, technically, death is not a clinical sign, this may be the main indication of an outbreak of colibacillosis in a flock (Barnes *et al.,* 2008). Clinical signs of predisposing or compounding factors often are seen concurrently with signs of *E. coli* infections (Barnes *et al.,* 2008).

2.5 Epidemiology and Disease Transmission

Avian colibacillosis is one of the prime causes of morbidity, mortality and decrease in productivity associated with heavy economic losses to the poultry industry, by its association with various disease conditions, either as primary or as a secondary pathogen (Barnes *et al.*, 2008; Singh *et al.*, 2011). It affects birds of all ages. Faeco-oral route is the main route of infection following ingestion of contaminated feed and water. Intestinal tract of animals, including poultry, is the most important reservoir of *E. coli.* Transmission of pathogenic *E. coli* through egg is common and can result in huge mortality in chicks. Pathogenic coliforms are more frequent in the gut of newly hatched chicks than in the eggs from which they hatched suggesting rapid spread after hatching (Adesiyun *et al.*, 2005). The most important source of egg infection seems to be faecal contamination of the egg surface with subsequent penetration of the shell and membranes (Chousalkar *et al.*, 2010). Coliform bacteria can be found in litter and faecal matter.

Pathogenic serotypes can also be introduced into poultry flocks through contaminated well water (Ozaki and Murase, 2009). Litter and fecal material are the source of coliforms, but *E. coli* forms the minor group (Nandi *et al.*, 2004). In the flock, environmental isolates constitute a separate and distinct population (Jeffrey *et al*., 2004). Dust may contain 10^5 - 10^6 *E. coli* per gram and the organism can be isolated from the environment even from the height of 40 feet outside the poultry house (Davis and Morishita, 2005). Contamination of feed as well as feed ingredients can introduce new serotype in the flock (Martins da Costa *et al.*, 2007). Rodent droppings may also be an important source. Gene transfer to susceptible strain from the resistant ones occurs in the mouse intestinal tract that provides suitable environment and is accelerated by the exposure to antibiotic (Hart *et al*., 2006). Contaminated well water may also act as an important vehicle of transmission (Morabito *et al.*, 2001). *E. coli* infection

is considered as a multifactorial disease. Environmental factors and immunosuppressive viral infections further influence the outcome of the disease. Virulence of *E. coli* along with host factors like initiation of egg production may be associated with the occurrence of colibacillosis in poultry (Someya *et al*., 2007). Increasing infection pressure in the environment increases the risk for colibacillosis. Infectious bursal disease (IBD), mycoplasmosis, coccidiosis, Newcastle disease or infectious bronchitis, as well as nutritional deficiencies all predispose the birds to this disease (Barnes *et al.*, 2008; Singh *et al*., 2011; Gowthaman *et al.*, 2012). The main reasons for the flare up of this commensal bacterium into a disease causing pathogen in poultry are unfavorable housing climate like dry and dusty conditions, poor ventilation, overcrowding, contaminated water, inclement weather conditions and stress on the affected birds lead to economic losses. Other risk factors are the duration of exposure, strain virulence, breed, and bird's immune status. Damage to the respiratory system like with an excess of ammonia or dust causing deciliation of the upper respiratory tract, renders the birds more susceptible to APEC infections. Distance between poultry farms and the hen density are also important risk factors (Landman and Cornelissen, 2006).

E. coli serotypes inhabit intestines of animals including humans and infect mammals, birds thus having a cosmopolitan distribution. *E. coli* inhabit intestines of poultry at concentration up to 10^6 per gram. Higher numbers are found in younger birds. They are also commonly isolated from the upper respiratory tract, skin and feathers of the birds. Among normal chicken 10-15% intestinal coliforms belong to potentially pathogenic serotypes (Gomis *et al*., 2001).

Dust in poultry houses may contain 10^5 - 10^6 *E. coli* per gram. These persist for long periods particularly under dry conditions. Faecal contamination of egg may result from the

penetration of *E. coli* through the shell and may spread to the chickens during hatching, often associated with high mortality rates, or leads to yolk sac infection. In a single bird a large number of different *E. coli* types are present, obtained via horizontal contamination from the environment, more specifically from other birds' faeces, water and feed. Rodent dropping also contain coliforms and thus are major source of infection to poultry (Antao *et al*., 2008).

2.6 Pathogenesis

Escherichia coli enter host tissues following mucosal colonization or directly through breaks or openings in the skin. Mucosal colonization is dependent on adhesin factors that permit the bacterium to attach to receptors and subsequently reproduce. A variety of fimbrial and non-fimbrial adhesins are produced by *E. coli,* which facilitates their attachment to host cells. Good evidence exist that two fimbriae— Type 1 (F) and P fimbriae— are important in the initial stages of infection. Type 1 fimbriae are expressed by *E. coli* that attach to upper tracheal epithelium (Pourbakhsh *et al.,* 1997), oviductal epithelium (Monroy *et al.,* 2005), and digestive tract mucosae (Edelman *et al*., 2003). P fimbriae are expressed in deeper tissues ((Pourbakhsh *et al.,* 1997). Type 1 fimbriae bind to mucus in the digestive tract but not to goblet cells producing the mucus. Flagella aid in penetrating the mucous layer in order to reach the cell surface, and curli, another adhesin factor, aids in attachment to the cell surface (La Ragione *et al.,* 2000).

Virulent strains are capable of traversing the mucosa, especially if an injurious agent has compromised it, and surviving within the internal milieu of the body. Air sac epithelial cells round up following inoculation with virulent strains, which causes them to separate from each other providing bacteria access to systemic tissues (DeRosa *et al.,* 1992). Toxins in cell-free culture filtrates, most likely endotoxin, produce the same acute inflammatory response as the living organism (DeRosa *et al.,* 1992).

Once *E. coli* becomes extra-mucosal, the environment it has entered is extremely hostile. Unless the organism is equipped with survival capabilities (*e.g.*, "virulence" factors), it is rapidly destroyed by phagocytic cells such as heterophils, thrombocytes, and macrophages (Harmon and Glisson, 1989; Harmon, 1998; Wigley, *et al.*, 1999). Macrophages located primarily in the spleen and liver phagocytize bacteria that gain access to the circulation (Arp and Cheville, 1981). Complement and antibodies to O antigens (endotoxin), outer-membrane proteins (siderophores), and fimbriae serve as opsonins to promote phagocytosis and destruction of the organism (Arp, 1982). Endotoxin also decreases the bacteriocidal ability of pulmonary macrophages (Emery *et al.*, 1991), which may aid in survival and dissemination of *E. coli.*

2.7 Disease Manifestations

The morbidity rate varies and mortality rate is around 5 – 20 %. Maximum mortality occurs within 5 days of onset of disease (Singh *et al.*, 2011). In birds, pathogenic *E. coli* infections may cause the following disease conditions (Singh *et al.*, 2011).

2.7.1 Colisepticaemia

Colisepticaemia the commonest infectious disease of farmed poultry seen worldwide in chickens, turkeys, etc. The bird gets infected mainly by inhalation of dust contaminated with faecal material. Infection can also occur by the oral route via water, feed and fomites, contaminated shell membranes or yolk. Young growing broilers (5 – 10 week) are mostly affected resulting in a mortality rate of 5 – 10 %, occasionally can reach 50 – 100 %. Air-sacculitis is seen in 0.5 - 2.5 % cases, with thickened and cloudy appearance of the air sacs. Surviving chicks are weak and stunted. Diarrhoea, pasty vent, loss of appetite, depression, dyspnoea and sneezing are the clinical signs seen in colisepticemia (Ewers *et al*., 2003 Vandekerchove *et al*., 2004b; Singh *et al.*, 2011).

2.7.2 Respiratory tract infection - Air sac disease (chronic respiratory disease)

Air-sacculitis is observed at all ages. *E. coli* occurs as a secondary invader in infectious bronchitis (IB), Newcastle disease (ND) and Mycoplasma infections, where it aggregates to air-sacculitis. Due to stress and high ammonia concentrations in litter, deciliation of trachea occurs and organisms get entry via inhalation in a dusty and overcrowded environment, and causes air sac infection. It occurs in growers of 6-9 weeks of age. In this infection, the air sacs become cloudy, oedematous, thickened and caseous deposition is observed. Histopathologically, single cell membrane becomes multilayered, oedematous with heterophil infilteration (Ginns *et al*., 1998; Al Ankari *et al*., 2001; Barnes *et al*., 2008).

2.7.3 Pericarditis and Perihepatitis

These conditions are also found in colisepticemia. Such conditions occur as a sequel to septicaemia and organism gets settled in liver and heart. Heart shows thickening of pericardium, which appears cloudy and filled with thick yellow (milky) pericardial fluid. Liver shows thick fibrinous membranous covering which appears very prominent and can be easily peeled off (Barnes *et al*., 2008).

2.7.4 Mushy Chick Disease

It is also known as yolk sac infection, sleeping disease, omphalitis, and is responsible for heavy early chick mortality. Neonatal infection of chicks can occur horizontally from the environment, or vertically from the hen. The chick can be infected during or shortly after hatching. Retained infected yolk, omphalitis, septicemia and mortality of the young chicks up to an age of three weeks can be seen. This occurs worldwide in chickens, turkeys and ducks due to bacterial infection of the navel and yolk sac of newly hatched chicks as a result of contamination before healing of the navel. Chicks once born stagger about and look sleepy. They will be reluctant to move and tend to stay under the heat source. Chicks will show

dejection, loss of appetite, swollen abdomen, vent pasting, and diarrhoea. Post mortem lesions include enlarged yolk sac with congestion and abnormal yolk sac contents, viscid and thick yolk, which changes to yellowish green, sticky/watery emitting a very foul smell (Shah *et al*., 2004; Singh *et al*., 2011). Mortality follows with the worst of it occurring within the first couple of days of the chick's life. Chicks that survive the first few days will usually never be as strong or healthy as the rest of the flock. The infection is caused by contamination of a number of bacteria types that enter through the porous egg shell inside the hatchery incubator or before the egg is placed into the incubator. Incubation conditions (37 ^{0}C) are ideal for breeding bacteria as well as incubating eggs. Once the egg is infected, some of the harmful bacteria are capable of breaking down the yolk sac which causes secondary infection to move in (Montgomery *et al*., 1999).

2.7.5 Acute septicemia/infectious disease

It is seen in mature and growing chicken and turkeys. Septicemia also affects chickens of all ages, and has been mainly described in broilers. It is the most prevalent form of colibacillosis, characterised by polyserositis and causes depression, fever and often high mortality. Several routes of infection are possible: neonatal infections, infections through skin lesions, infection of the reproductive organs, of the respiratory tract and even infection *per os*. When *E. coli* reaches the vascular system, the heart and internal organs are infected.

Pulmonary congestion, green liver, congested pectoral muscles, small white foci in liver, enlargement of spleen and liver and a tendency towards pericarditis and peritonitis is seen. The infection of the myocardium causes heart failure. Birds die acutely, and on post mortem good flesh and full crop is seen. Septicemia occasionally also leads to synovitis and osteomyelitis and on rare occasions to panophthalmia (Pourbakhsh *et al*., 1997; Barnes *et al*., 2003, 2008; Singh *et al*., 2011).

2.7.6 Peritonitis

It occurs in laying hens and breeders. In the abdominal cavity of affected birds, fibrin and free yolk is observed in this condition. Acute mortality occurs. Bacteria enters from intestine to oviduct by anti-peristaltic movements, grows in yolk material deposited in peritoneal cavity and produces yellowish fibrinous or fibrinopurulent (pus like) material in abdominal cavity (Landman and Cornelissen, 2006).

2.7.7 Panophthalmitis

Panophthalmitis is an uncommon manifestation of *E. coli* septicaemia. In this condition, blindness may occur due to hypopyon and hyphema of eye. The eyes are swollen along with cloudy to opaque appearance with initial hyperemia. The presence of fibrinoheterophilic exudates as well as several bacterial colonies is characteristic. The inflammation may turn to granulomatous reaction gradually. There may be persistence of the organism in the affected eye for long with characteristic outcomes like retinal detachment as well as retinal atrophy and lysis of the lens. Most birds die shortly after onset of lesions, some may recover. Histopathologically, heterophil infiltration and formation of giant cells around necrotic areas is observed (Nakamura and Abe, 1987; Barnes *et al.,* 2003).

2.7.8 Synovitis

This is joint inflammation, which occurs as a sequalae to colisepticemia and is experimentally produced by intravenous inoculation of *E. coli.* Many birds recover within a week but others can become chronically infected and emaciated (Droual *et al*., 1996).

2.7.9 Salpingitis

Salpingitis, an inflammation of the oviduct, results in decreased egg production and sporadic mortality in laying chickens and others like duck and geese. The oviduct may contain big

caseous mass. Layers as well as broilers may suffer from acute or chronic salpingitis, resulting from an ascending infection from the cloaca or an infection of the left abdominal airsac (Landman and Cornelissen, 2006; Ozaki and Murase, 2009). Affected birds cannot produce and lay eggs. In chronic salpingitis, the oviduct has a yellowish-gray, cheese-like content, with a concentric structure. Hisopathologically, the tissue reaction in the oviduct is mild, consisting largely of multifocal to diffuse heterophil accumulations subjacent to the epithelium. In layers, salpingitis can cause egg peritonitis if yolk material has been deposited in the peritoneal cavity, characterized by a sero-fibrinous inflammation of the surrounding tissues. A laying hen suffering from *E.coli*-induced oophoritis or salpingitis may infect the internal contents of the egg before shell formation completes (Jordan *et al.,* 2005; Timothy *et al*., 2008).

2.7.10 Bumble foot (ulcerative pododermatitis)

This is a bacterial infection and inflammatory reaction on the feet of birds and rodents and is much more likely to occur in captive animals than in those in the wild. It is caused by *E. venezuelensis* and *E. coli*. It is a common infection for domesticated poultry and waterfowl such as chickens and ducks. Due to constant walking on hard, rough, or sharp surfaces, birds can develop small wounds on bottom of their feet. Symptoms of bumble foot are limping and a large soft swelling on bottom of foot. Usually bumble foot is due to bruises or small wounds infected with bacteria. *E.coli* and *Staphylococcus aureus*, both produce this condition in chicks (Gross *et al*., 1994).

2.7.11 Necrotic dermatitis (cellulitis)

Broilers may be affected by necrotic dermatitis, also known as cellulitis, characterized by a chronic inflammation of the subcutis on abdomen and thighs (Kumor *et al*., 1998).

2.7.12 Swollen Head Syndrome (SHS)

It is an acute to sub-acute cellulitis involving the periorbital and adjacent subcutaneous tissues of the head. It is mainly a problem in broilers, causes oedema of the cranial and periorbital skin. Microscopic lesions include fibrino-heterophilic inflammation and heterophilic granulomas in the air-spaces of the cranial bones, middle ear and facial bones; lymphoplasmacytic conjunctivitis and tracheitis with formation of germinal centres. Affected birds show ocular discharge and conjunctivitis progressing to periorbital swelling. Terminally, eyes are closed and enlargement of the head is a prominent sign in severely depressed or recumbent broilers. Acutely affected birds may show tracheal hyperaemia and pulmonary congestion. Avian pneumovirus (APV), turkey rhinotracheitis (TRT) virus along with *E. coli* is associated with SHS, considered as a disease caused by usually followed by an opportunistic *E. coli* infection. SHS can cause a reduction in egg production of 2 to 3 %, and a mortality of 3 to 4 % (Gross, 1990; Van de Zande *et al*., 2001).

2.7.13 Infectious Asthenia

It is enteritis occurring in broilers (adults) and breeders of 10 weeks or greater age, occurring as sporadic outbreaks. Great emaciation and weakness, sitting on haunches, inflammation of duodenum, wasting of muscles of breasts and legs is observed and birds show yellowish diarrhoea (Jordan, 1990).

2.7.14 Hjarre's Disease (Coligranuloma)

Hjarre's Disease is a rare form of systemic colibacillosis that affects adult chicken and turkey (Nolan *et al.*, 2013), characterized by nodular granulomas in liver, mesentery and walls of intestine, gizzard, duodenum, mesenteries, caeca, lungs and kidneys. Nodules are not observed in spleen. Coligranuloma is a relatively uncommon coliform disease, a rare form of colibacillosis, mostly seen at necropsy (1/10,000 cases), and may cause mortality as high as

75 % in an individual flock. Pathological lesions includes coagulative necrosis and enlargement of liver, hard nodular granulomas in the mesentery, walls of intestine, particularly caecum, congested and swollen spleen with abnormal contours, congested viscera with atrophied bursa, normal peripheral nerves and bone marrow. Nodules are millet seed sized, like in tuberculosis (Shah and Qureshi, 2006; Ewers *et al*., 2007).

2.8 The Poultry Industry

In Nigeria, before and immediately after independence, agriculture was the main-stay of the economy (Akangbe *et al.,* 2012). However, its contribution to the economy has been declining since the oil boom of the 1970's. Toluwase and Akpata (2013) reported that agriculture in the post independent years was the main stay of the Nigeria economy but suffered neglect due to the oil boom of the 1970's.

The Nigerian poultry industry in particular has been rapidly expanding in recent years and is therefore one of the most commercialized (capitalized) subsectors of Nigerian agriculture (Adene and Oguntade 2006). The popularity of poultry production can be explained by the fact that poultry has many advantages over other livestock. Poultry birds are good converters of feed into useable protein in meat and eggs. The production costs per unit remain relatively low, and the return on investment is high. Therefore, farmers need a relatively small amount of capital to start a poultry farm (Heise *et al.*, 2015). Furthermore, poultry meat is very tender and acceptability to consumers is high, regardless of their religious beliefs. Also, the production cycle is quite short, so capital is not tied up over a long period. Finally, eggs, one of the major products of poultry production, are more affordable for the common person than other sources of animal protein (Ojo 2003; Aboki *et al.* 2013). Poultry meat and egg accounted for about 30% of the total livestock output in Nigeria, of which eggs accounted for

over 80% (Evbuomwan, 2006). Eggs and poultry meat has emerged next to milk as a contributor to the output from livestock sector in recent years (Ekunwe and Soniregun, 2007).

2.9 Female Reproductive System

The female reproductive system remains dormant in the young chicken and growing pullet until she reaches the age when these organs start to prepare for the normal production of eggs. One of the first signs of her developing maturity is the change in the comb development. This organ starts to grow and to take on a vivid red hue as the hormones produced by the now awakening ovary start to have an effect. (Sheerwood *et al.,* 2005).The avian gonads arise from more than one embryonic source. The medulla or core arises from the mesonephric ducts. The outer cortex arises from a thickening of peritoneum along the root of the dorsal mesentery within the primitive gonadal ridge. Mesodermal germ cells that arise from yolk-sac endoderm migrate into this gonadal ridge, forming the ovary. The cells are initially distributed equally to both sides. In the hen, these germ cells are then preferentially distributed to the left side, and migrate from the right to the left side as well (Pollock and Orosz, 2002).

The female reproductive system in the domestic fowl consists of the ovary and the accompanying oviduct. While the female embryo chicken has two sets of reproductive organs, only one of these, the left survives and reaches maturity to produce eggs. The single surviving ovary is located in the laying hen just in front of the kidneys in the abdominal cavity and is firmly attached to the wall of the cavity. The ovary is well endowed with blood vessels to ensure there is no hindrance to the transport of nutrients to the developing yolk (Pollock and Orosz, 2002; Sheerwood *et al.*, 2005).

2.9.1 Oviduct

The function of the oviduct is to produce the albumen, shell membranes and the shell around the yolk to complete the egg. It is a long tube well supplied with blood via numerous blood vessels. There are many glands found in its walls that produce the albumen, the shell membranes and the shell. In the non-layer the oviduct is quite short and small in diameter. However, once the reproductive system becomes active, it grows to a length of 70 - 80 centimetres with a variable diameter depending on the function of the section being examined.

The oviduct consists of five distinct parts or sections, each having different functions (Bradley, 1960; Parkhurst and Mountney, 1988; North and Bell, 1990).

The infundibulum is a muscular funnel-shaped structure marking the beginning of the oviduct. It is in close proximity to the growing follicles and surrounds them so that the ovulated follicle is trapped and directed to descending parts of the oviduct. The infundibulum has no secretory functions and the egg remains here for a period of 15 to 17 minutes. However, if the infundibulum malfunctions, the follicle may ovulate outside of oviduct into the internal peritoneal cavity. A study conducted on Brown-Leghorn pullets revealed that when this 'internal laying' was evident there was a loss of 12% of potential eggs (Wood-Gush and Gilbert, 1970).

The magnum is the longest part of the oviduct and contributes the egg white protein to the developing egg during its 3 hours of passage through this segment. Synthesis of albumen largely occurs continuously in the tubular gland cells of magnum; however, deposition rate is heightened when the egg is present in this region (Muramatsu *et al*., 1991).

Albumen provides an antimicrobial function prior to egg incubation, and its proteins are the major source of nutrition for the growing embryo during incubation (Willems *et al*., 2014).

The isthmus bridges the magnum and the shell gland in the chicken oviduct. When the inchoate egg passes through the isthmus, the eggshell membranes (ESM) are secreted and assemble around the egg white. Besides covering the inner soft structures, the ESM provides a structural foundation for eggshell calcification. The ESM is a clear film primarily composed of fibrous proteins such as collagen. The expression of COL10A1, FBN1, CREMP, LOX, TXN, QSOX1 in the isthmus are involved in the formation of ESM (Corson *et al*., 1993; Kodali *et al*., 2011; Du *et al*., 2015,). However, the mechanism of formation of ESM in the isthmus is not completely understood.

The vagina is the caudal portion of the chicken oviduct and does not play a role in egg formation. However, this is where the outer cuticle forms and pigmentation occurs. The muscles of the vagina help to turn the egg longitudinally to the blunt end and push the egg out of the oviduct.

2.9.2 Avian ovary and ovarian follicles

The ovary consists of a mass of yellowish, rounded objects called follicles, each containing an ovum or yolk. There are many such follicles but only a small number in comparison will ever reach maturity to produce an egg. When the hen is in lay the ovary will be active. The size of the follicles will vary from very small to those approaching the normal yolk size in the egg which can be up to 40 millimetres in diameter, and will contain a fully matured yolk ready for release into the oviduct.

It is possible to find five stages of development in the active ovary:

1. Primary follicles – follicles that have not yet commenced to grow
2. Growing follicles-follicles that have commenced to grow
3. Mature follicles – follicles ready or nearly so for release
4. Discharged follicles – where the yolk has just been released

5. Atretic follicles – those from which the yolk has been released some time ago.

It takes approximately 10 days for a yolk to develop from the very small to the normal size found in eggs and during this time it is contained in the follicle. The follicle acts as a sack during this period of development supplying it with the nutrients required for its growth. When a mature follicle is examined an elongated area virtually free of blood vessels will be found on the distal surface of it. This area, called the stigma**,** is where the follicle normally splits to release the yolk into the oviduct. At any one time follicles of various sizes, containing yolks at different stages of development can be found in the ovary (Taylor, 1970). If, for some reason, the follicle splits at other than the stigma, the numerous blood vessels that rupture will result in free blood being found in the egg that is a blood spot will form.

2.10 Collection of Blood Sample

Blood collected from a bird for haematology and serum biochemistry should be of venous origin and in most birds it can be taken from the basilic vein (wing or brachial vein) which cross the ventral surface of the humeral radio-ulnar joint (elbow) immediately under the skin or the jugular vein usually the right which is larger than the left and caudal tibial vein (medial metatarsal which is located on the medial side of the tibiotarsus above the tarsal joint, sample can be collected from the most species of birds under manual restraint in the dorsal position (Samour, 2004). The wing vein is superficial and easily visualized in adult birds and chickens and poults older than 1.5 - 2 weeks of age.

Birds that weigh less than 500 g usually are sampled with a 25 gauge hypodermic needle and a 1 or 2ml syringe, whereas a 23 gauge hypodermic needle is best used for birds of more than 500 g (Cooper, 2002). Veins of very small birds can be nicked with a scalpel blade and blood collected in a capillary tube (Dawson and Bortolotti, 1997). Butterfly catheters may lessen the effect of bird movements during sampling (Cooper, 2002). Smaller gauge needles

increase the risk of haemolysis. The use of larger needles increases the risk of hematomas (Fudge, 2000). Excessive negative pressure may collapse veins (Jennings, 1996). Regardless of technique, the phlebotomy site must be prepared aseptically to prevent bacterial contamination (Fudge, 2000). Both wings can be used and collection can be performed with or without assistance. To prevent flapping and kicking, control of unused wing and feet is essential. This is achieved by holding the bird in one arm with the birds feet focusing behind the upper arm towards the rear pinning the unused wing between the birds body and collectors trunk and out stretching the target wing with the holding arm. The birds head is generally held down which has a calming effect. This method leaves the collector one hand free to collect the blood but also can be used when assisting others (Wakenell, 2010).

Cardiac puncture is used in a situation where birds will be euthanized immediately after the procedure and is commonly used for assessing maternal antibody level in a newly hatched chicken. Due to the risk of damaging the lung and expectoration of blood, this technique must only be used by trained personnel if birds are not euthanized (Wakenell, 2010).

The average blood volume of most birds is approximately 10 % body weight. Ten percent of this volume may be removed from circulation for testing (or 1% of the bird's body weight) for example, up to 3ml may be collected from 300 mg parrot (Campbell, 2000).

2.11 Haematology

2.11.1 Erythrocytes

Avian erythrocytes are oval, nucleated cells, with occasional round immature form. Mature chicken and turkey erythrocytes found in the peripheral blood are large elliptical cells measuring approximately 12 x 6 μm. They have homogenous eosinophilic cytoplasm and a central round to oval nucleus with a condensed chromatin pattern. Because *erythropoiesis* is intravascular or *intrasinusoidal,* occasional *rubricytes* can be found in the peripheral blood of healthy birds (Wakenell, 2010). Erythrocytes are the most common cells in avian (and other

non- mammalian) blood. In all non-mammals, the erythrocyte of the circulation is a true cell; complete with nucleus. As in mammals, the function of the erythrocyte is to carry oxygen to the tissue (Rosalie, 1991). The percentage of *reticulocyte*s seen in the peripheral blood of normal chickens and turkeys is somewhat higher than in most mammals. *Polychromasia* is more prominent in younger birds, but typically does not exceed 5%. Common artifactual abnormalities in erythrocyte morphology include cytoplasmic refratile vacuoles, smudge cells and various morphologies brought about by stretching cells while preparing the smear such as splinding, bilobbed nuclei, bare nuclei and *erythroplastids* (Anucleate fragments of erythrocyte cytoplasm) (Wakenell, 2010).

Erythrocyte concentration, packed cell volume (PCV), and haemoglobin concentration (Hb) may be influenced by age, gender, hormones, and other factors. Packed cell volume and total erythrocyte count tend to be higher in male birds than in female birds and also tend to increase with age (Herbert *et al.,* 1989). The normal PCV for many bird species ranges approximately between 35% and 55% (Thrall, 2004).

Avian erythrocytes have a shorter half-life, (ranging from roughly 25 to 45 days in various species) than that of many mammals. Because erythrocyte turnover is more rapid, birds tend to have higher percentages of polychromatophils in health than mammals. Avian erythropoietin is a glycoprotein that is synthesized in the kidneys and stimulates bone marrow erythropoiesis. There is apparently no cross-reactivity between avian and mammalian erythropoietin (Herbert *et al.,* 1989).

Several erythrocyte parameters, including RBC count, may be measured quantitatively on impedance-based or flow cytometric haematology analyzers with appropriate adjustments for other nucleated cells. Manual methods of counting avian erythrocytes are well described, and are often implemented in practice, given the paucity of automated instrumentation

appropriate for use in avian haematology. The PCV can easily be obtained by means of centrifugation of a filled microhaematocrit tube. Haemocytometer counting of erythrocytes can be accomplished using the Unopette (Becton-Dickinson, Rutherford, New Jersey) method or using Natt and Herrick's solution (Natt and Herrick, 1952).

The erythrocyte count can be obtained using either a standard manual method or an automated method, such as those used for mammalian blood. The packed cell volume is determined using the standard microhaematocrit method with centrifugation at 12,000 *g* for 5 minutes. The haemoglobin concentration is best determined using the cyanmethemoglobin method following proper removal of free red cell nuclei by centrifugation.

The mean corpuscular values (or the RBC indices) are blood cellular indices that provide information about the haemoglobin content and size of RBC. It include the average red blood cell size (MCV), haemoglobin amount per red blood cell (MCH), and the amount of haemoglobin relative to the size of the cell (Hb) per red blood cell (MCHC). These parameters can be calculated using the standard formulae once the red blood cell count, PCV, and Hb have been obtained. These RBC measures are used to diagnose the presence of anaemia and the types of anaemia (Campbell, 2004a).

More immature avian erythrocytes are round cells with basophilic cytoplasm, round nuclei, and more open nuclear chromatin; elliptic erythrocytes develop approximately at the reticulocyte or polychromatophil stage (Mitchell and Johns, 2008). Immature erythrocytes, especially the rubricyte stages often appear smaller than mature erythrocytes and are round to slightly oval. The cytoplasm of immature erythrocytes have an irregular cytoplasmic polychromasia and stains more basophilic than mature erythrocytes and a more rounded, pale nucleus (Campbell and Dein, 1984; Campbell, 1988; Campbell, 2004b). Early immature erythrocytes (rubriblasts and prorubricytes) are rarely seen in peripheral blood films of birds

and their presence may indicate a marked erythropoietic response or erythrocyte dyscrasia such as erythroblastosis. Birds suffering from heavy metal toxicosis, especially lead poisoning, often reveal an inappropriate release of immature erythrocytes in a non-anaemic patient. This is reflected in the peripheral blood film by two distinct populations of erythrocytes, immature cells (i.e. metarubricytes and polychromatic erythrocytes) and old mature cells with pyknotic nuclei in the blood films from lower vertebrate patients, it is relevant to take note of the number of immature erythrocytes. Animals that are responding to anaemia may exhibit an increase in the number of immature erythrocytes in the peripheral blood film (Campbell, 2004a).

The erythrocyte morphology, including size variation (anisocytosis), shape abnormalities (poikilocytosis), and abnormalities in haemoglobinization, can be evaluated in the monolayer region of a well made smear. Semi-quantitative estimates of polychromasia, anisocytosis, poikilocytosis, and degree of erythrocyte parasitism (if present) can be performed (Mitchell and Johns, 2008).

2.11.2 Leukocytes

Total leukocyte counts with differential leukocyte counts are part of the basic patient profile sought for inevaluation of ill animals, and of routine evaluation of healthy animals. They influence the diagnostic work up and treatment (Aroch *et al.,* 2013). The leukocytes consist of granulocytes (named for their conspicuous cytoplasmic granules) which are the heterophils, eosinophils and basophils) and the agranulocytes (lymphocytes and monocytes) which are the mononuclear leukocytes (Lucas and Jamroz, 1961; Mitchell and Johns, 2008). In non-mammalian species generally differentiating between eosinophils and heterophils, small lymphocytes and thrombocytes, and large lymphocytes and monocytes is often difficult (Latimer and Bienzle, 2010). In birds species with published values of haematological

parameters, however, only lymphocytes and heterophils are detected in sufficient numbers to enable reliable inter-individual comparison (the combined number of lymphocytes and heterophils typically accounts for 85 – 95 % of all leukocytes in a blood smear) (Davis, 2009). The differential leukocytes count in non-mammalian species is performed by counting 100 - 200 leukocytes in stained blood smears, and has been studied mostly in the domestic chicken (*Gallus gallus*) (Campbell, 2004b).

The total leukocytes count in non-mammalian vertebrates can be counted using three methods;

1) direct haemocytometer counting with Natt and Herrick's or toluidine blue stain solutions (Natt and Herrick, 1952; Lane, 1991; Robertson and Maxwell, 1993; Campbell, 2004b);
2) semi-direct haemocytometer count with phloxine-B dye or the (now discontinued) commercial eosinophil Unopette 5877 (Becton - Dickinson, Rutherford, NJ) (Natt and Herrick, 1952; Costello, 1970; Lane, 1991; Campbell, 2004b; Campbell, 2010; Wakenel, 2010), combined with manual differential leukocytes count in Romanowsky-stained blood smears (Ferris and Bacha, 1984; Zinkl, 1986; Latimer and Bienzle, 2010);
3) Semi-quantitative total leukocytes count evaluation in Romanowsky stained blood smears, which is considered less accurate (Campbell, 2004b; Wakenel, 2010).

The latter can be done by averaging leukocytes number in 10 monolayer, X400 fields and multiplying it by 2,000, yielding the total leukocytes count in cell/µl. Alternatively, one can determine the leukocytes average number in five X 1,000 monolayer fields, multiply it by 3,500,000 (the approximate number of erythrocytes in 1 µl of blood in birds when the PCV is 35 – 55 %), and dividing the result by 1,000 (the average number of erythrocytes in 5 X1,000

monolayer fields). The result should be corrected if PCV is abnormally high or low (Zinkl, 1986; Campbell, 2004b; Wakenel, 2010).

Direct leukocytes count is a complex, time-consuming procedure, involving preparation of the stain solution, and is complicated by the need to differentiate lymphocytes from thrombocytes, and with presence of stained erythrocytes in the haemocytometer (Zinkl, 1986; Campbell, 2004b; Latimer and Bienzle, 2010). The semi-direct counting method relies on the positive staining of heterophils and eosinophils by the dye, allowing their counting in the haemocytometer. Calculating their total number per microlitre, and then the total leukocytes count is achieved by performing a differential leukocytes count using a Romanowsky-stained blood smear. This method is more precise and less time consuming than the direct haemocytometer count (Zinkl, 1986; Campbell, 2004b; Campbell, 2010; Wakenel, 2010); however, it becomes progressively less accurate for estimation of the total leukocytes count with increasing proportion of mononuclears to granulocytes (Campbell, 2004b). Currently, the Unopette 5877 kit has been discontinued, while the other staining solutions mentioned are not readily available in most veterinary clinics. In contrast, Romanowsky-based quick staining solutions are readily available and are used in many veterinary clinics for blood smear staining. Based on the staining affinity of heterophils and eosinophils, eosin-based staining solutions are expected to stain these cells similarly as phloxine-B (Aroch *et al.*, 2013).

It is worth noting that the direct haemocytometer counts are more accurate than WBC estimates determined by the Unopette system method because of the variation in differential counts (Russo *et al.*, 1986). Both are subject to the technical error of the haemocytometer method, and changes in the WBC count may be caused by the variability inherent in the method of enumeration (Russo *et al.*, 1986). Part of this error may be decreased by

standardizing the technique (using the same chamber, cover slip and pipette and having the same person perform the counts) (Russo *et al.*, 1986).

2.11.2.1 Heterophils

The avian heterophils which is mostly compared to that of the neutrophils in mammals is the most frequently seen granulocyte in all species. As with neutrophil, the heterophils are instrumental in body defenses with incredibly large number available as well as their ability to phagocytes foreign bodies' bacteria (Rosalie, 1991). However, there are differences in granule contents and response to some stimuli. It has been speculated that avian heterphil either do not contain or contain minimal quantities of species lysozome. Heterophils lack myeloperoxidase and alkaline phophatase. Chicken and turkey hetrophils, unlike neutrophils, do not respond to stimulation by formylmethionyl-leucylphenylalanine (FMLP) and then production of oxygen radicals is lower than that of mammalian neutrophils (Ramirez, 2004). In Wright stained preparations of the avian blood smears, mature heterophils are medium sized round shaped cells with a lobed basophilic nucleus (usually 2 to 3 lobes) with densely clumped chromatin and prominent eosinophilic (orange red to red brown or pale blue) cigar to rod shaped granules. However, there is significant variation among bird species in the shape of the cytoplasmic granules (Mitchell and Johns, 2008). Specific granules are elliptical, although in some species they may be oval or rounded and have a distinct central body that appears refractile (Campbell, 2004b). The cytoplasm of heterophils is generally colourless (Mitchell and Johns, 2008). Heterophils are the most common granulocytes in circulation in the majority of birds (Mitchell and Johns, 2008; Claver and Quaglia, 2009). The response of heterophils to infections is similar to that of the mammalian neutrophil, migrating to the sites of inflammation caused by any offending pathogen, hence, killing it (Vegad and Katyar,

1995). Owing to their highly phagolytic activities, they are capable of a broad spectrum antimicrobial activity (Claver and Quaglia, 2009).

There are 2 types of changes observed in heterophils during the course of disease processes in birds. One change is the presence of immature cells in the peripheral blood, representing recruitment of cells from the bone marrow in response to cytokines and other inflammatory mediators. These immature cells (usually myelocytes and metamyelocytes) have more basophilic cytoplasm than mature heterophils. They have segmented nuclei and fewer specific granules that are occasionally immature (i.e. primary granules) (Campbell and Ellis, 2007).

Band heterophils are similar to mature heterophils, except the nucleus is horseshoe shaped with parallel sided and lacks lobes. Often the nucleus is obscured by the cytoplasmic granules. Metamyelocytes and myelocytes are less mature cells and are larger than the band heterophils. The nucleus of these cells is round to oval, and the cytoplasm is basophilic. Myelocytes and metamyelocytes have spiculate cytoplasmic granules; however, in myelocytes, the granules take up less than one-half of the cytoplasm, whereas in metamyelocytes, the granules take up more than half of the cytoplasm (Campbell and Ellis, 2007).

Band heterophils are identified in peripheral blood smears in the first 12 – 24 hours after the initial insult with persistence of leucocytosis for 7 days (Latimer *et al.,* 1988). However, the presence of immature cells in avian blood smears indicates acute inflammation. A degenerative left shift, in which the number of immature heterophils exceeds the number of mature heterophils indicates intense tissue demand for cells and carries a poor prognosis (Mitchell and Johns, 2008). Typically, when immature heterophils are found in the peripheral blood, normal appearing mature heterophils can be found (Campbell, 2004b).

The other change observed in avian heterophils during disease is toxic change which is similar to those observed in mammalian neutrophils. Generally, when toxic heterophils are seen, all the heterophils in the film appear toxic and usually to the same degree unless the condition is caught in the per-acute stage or is resolving (Campbell, 2004a). Toxic heterophils are classified on a scale of +1, +2, +3 and +4 depending upon the degree of toxicity. A +1 toxic heterophil shows increased cytoplasmic basophilia. A +2 toxic heterophil shows increased cytoplasmic basophilia, slightly abnormal granulation (i.e. partial degranulation, coalescing granules, or abnormal appearing granules or vacuolation). A +3 toxic heterophil will show changes that are more severe than +2 toxicity and the nucleus may show slight karyorrhexis or karyolysis. A +4 toxic heterophil will show marked changes in the cytoplasm and nucleus. Toxic heterophils are uncommon and usually seen in birds that are critically ill (Campbell, 2004a).

Avian heterophils are involved in controlling bacterial, viral and parasitic infections. One striking difference between birds and mammals is the process of pus formation. In mammals, neutrophils accumulate, leading to liquefaction and abscess formation. This liquid pus can spread along tissue planes or can form exudates that are removed by way of clearance pathways such as mucociliary apparatus (Harmon, 1998). In birds, however, heterophils accumulate and are resolved by means of inspissations of necrotic heterophils into a caseous mass rather than liquefaction. The necrotic heterophils are walled to form heterophilic granulomas (Montali, 1988; Harmon, 1998). The process is advantageous except when the caseous masses that are formed interfere with organ functions, such as in granulomas in the lungs or air sacs. In certain locations, these caseous masses could persist indefinitely. The exact mechanism of pus formation in birds has not been completely understood, though proposed mechanisms include variances in hydrolytic enzyme activity, such as lack of

myeloperoxidase in heterophil, or the lack of as yet-to-be identified proteases in birds (Harmon, 1998).

Conditions that cause an increase in the peripheral blood level of heterophils include infection (e.g. bacterial, fungal, viral, parasitic), inflammation, stress, certain toxicities, trauma, and leukaemia (Gildersleeve *et al.*, 1987; Andreasen *et al.*, 1993; Harmon, 1998; Bienzle and Smith, 1999). Infectious agents that commonly lead to heterophilia include *Mycobacterium* spp, *Chlamydophilia psittaci*, *Aspergillus* spp and birds acutely or chronically infected with *Mycoplasma* spp. Heterophilia associated with infections with these organisms is commonly accompanied by monocytosis (Branton *et al.*, 1997).

Heterophilis have been demonstrated to be the dominant component of exudates in birds, although lymphocytes and basophils may also be present early in inflammation (Harmon, 1998). Heterophilia with toxic change is indicative of severe systemic illness such as septicaemia, chlamydophilosis, fungal infection, or viraemia. The development of toxic change may indicate lack of control of an infectious process and often carries a poor prognosis (Campbell and Ellis, 2007).

Some toxins (e.g. organophosphate) can lead to heterophilia (Heatley and Jowett, 2000). In addition, heterophilia has been observed in cases of zinc toxicosis, presumably as a result of gastrointestinal inflammation, stress, and decreased resistance to pathogens (Campbell and Ellis, 2007).

Similar stress response as seen in mammals has been reported in birds. For example, Macaws may demonstrate marked leucocytosis with heterophilia as a result of transport and handling. Corticosteroid administration results in an increase in circulating heterophils and lymphopaenia in birds (Harmon, 1998).

2.11.2.2 Eosinophils

Eosinophils are round to irregular shaped and are approximately 12 μm in diameter. They have a lobed nucleus and light blue cytoplasm with eosinophilic round to oval granules that stain brighter than those of heterophils and lack central body. Immature heterophils have round granules and could be confused with eosinophils. Cytochemical stains can distinguish between the two types of cells. Eosinophils are positive for peroxidase and acid phosphatase activity and Sudan black B while heterophils are negative (Ramirez, 2004). Eosinophils are typically associated with allergic reaction and parasitic infections and rarely seen in high number except in raptor species (Samour, 2004).

Eosinophilia has been observed after foreign antigen administration and possibly in association with alimentary tract parasitism (Montali, 1988; Latimer and Bienzle, 2000), though parasite antigens do not generally induce eosinophilia in birds (Montali, 1988). The parasitic response by this cell type in birds has not been scientifically confirmed (Montali, 1988; Latimer and Bienzle, 2000; Cherry, 2008).

Other studies have shown eosinophilia with generalised inflammation in birds. It is possible that avian eosinophils play a role in delayed hypersensitivity, but they have been shown to be related to anaphylaxis or other acute hypersensitivity reactions (Montali, 1988). Severe eosinophilia have also been observed in cases of poxvirus infection in red-tailed hawks, although the mechanism of this response in unknown (Mitchell and Johns, 2008).

2.11.2.3 Basophils

The basophil is a round cell with deeply basophilic granules in the cytoplasm and a round light blue, central, non-indented nucleus. It resembles the mammalian mast cell. Birds appear to have more mast cells and basophils than mammals. The basophil measures approximately

12μm in diameter and the granules often partially obscure the nucleus. These granules may dissolve or condense in Wrights stains (Wakenell, 2010).

Basophils appear to play important role in the initial phases of acute inflammation and immediate hypersensitivity reactions, but differ from those in mammals by not contributing to delayed hypersensitivity. This, however, does not always result in peripheral basophilia (Montali, 1988; D'Aloia *et al*., 1994; Maxwell and Robertson, 1995; Campbell and Ellis, 2007).

The granules of avian basophils contain histamine, as in mammals (Campbell and Ellis, 2007). Therefore, it is suggested that they function in acute inflammatory and type IV hypersensitivity reactions, similar to mammalian basophils and mast cells (Mitchell and Johns, 2008). Severe stress has also been proposed as an underlying cause for an increased basophilic response in birds (Maxwell, 1993; Altan *et al.*, 2003; Campbell and Ellis, 2007; Bedáňová *et al.*, 2007).

As avian basophil granules are extremely water soluble, they may be easily damaged during the staining process (Lucas and Jamroz, 1961), which impairs the detection of these cells. This might be the reason why precise data on the variability of basophil levels in peripheral blood are limited in free-living birds. Nevertheless, there is some clear evidence suggesting that the proportion of basophils among blood-borne leukocytes is much higher in some avian species than the normal physiological values of most mammals (Maxwell and Robertson, 1995).

2.11.2.4 Monocytes

Chicken and turkey monocytes are usually the largest leukocytes (approximately 14 μm in diameter) and need to differentiate from large lymphocytes. Monocytes are round cell usually had indented nuclei and abundant pale, vacuolated blue-grey cytoplasm. The cytoplasm

contains fine azurophilic granules. The monocyte is a pleomorphic cell and can be difficult to identify. It is important to be consistent with lymphocyte classification to aid in differentiation from monocytes. Lymphocytes are round and generally have less cytoplasm than monocytes, size is dependent on activation state in chickens and blood monocytes cannot be differentiated from lymphocytes solely on the basis of size (Wakenell, 2010). Monocytes are the largest leukocytes in normal peripheral blood smears, and they resemble their mammalian counterparts. The typical monocyte is round or amorphous in shape with a kidney-shaped, rounded, oval or lobed nucleus. The chromatin is lace-like and not as densely clumped as in lymphocytes. The cytoplasm is generally deep blue or grayish blue often presenting a fine pink- or purple-staining granular area near the nucleus and contains discrete vacuoles (Mitchell and Johns, 2008; Claver and Quaglia, 2009). High vacuolated monocyte suggests increased phagocytic activity and may indicate a response to a systemic antigen (Campbell, 2004a). Monocytes have more cytoplasm and a paler nucleus than large lymphocytes (Campbell and Dein, 1984).

Monocytes have phagocytic activity and transform into macrophages after they migrate into tissues. Their cytoplasmic granules contain lysozymes that are involved in the destruction of invading organisms and chemicals involved in mediating inflammation (Latimer and Bienzle, 2000; Mitchell and Johns, 2008).

Increased monocytes in peripheral circulation (monocytosis) are often seen with infectious and/or inflammatory disease, especially with granulomatous diseases such as aspergillosis or mycobacteriosis. *Chlamydophila psittaci* infections in birds also often results in monocytosis usually due to the production of chemotactic agents that attract monocytes (Campbell, 1994). Although monocytosis is common with chronic inflammation, acute infections, such as *Mycoplasma* spp infections, may lead to monocytosis in addition to heterophilia and

lymphopaenia (Branton *et al.,* 1997). Monocytosis has also been observed in birds fed a zinc-deficient diet (Wight *et al.,* 1980).

2.11.2.5 Lymphocytes:

Lymphocytes may be the major circulating leukocytes in the blood of some species of birds (Campbell and Dein, 1984). Avian lymphocytes are similar in morphology to mammalian lymphocytes. They are round cells with centrally positioned or slightly eccentric nuclei. The nucleus chromatin is densely aggregated and there is a high nuclear-to-cytoplasm (N/C) ratio. The cytoplasm is basophilic and homogenous, with no vacuolation. Generally, there are no granules in lymphocytes, although occasionally, rare azurophilic granules may be present. There is significant variation in the sizes of the avian lymphocytes. Small lymphocytes often resemble thrombocytes (Mitchell and Johns, 2008). Small lymphocytes predominate in most birds, with irregular projections or blebs frequently observed on this cell type (Campbell, 1994). The large lymphocytes may be difficult to distinguish from monocytes. Large B lymphocytes, that is, plasma cells are sometimes seen in avian blood smears. These cells are large with eccentrically located nuclei, an intensely basophilic cytoplasm, and a distinct Golgi zone (Mitchell and Johns, 2008).

Birds have been used as pioneer model to study immunology, which resulted in the identification of a B- and T-lymphocyte lineage. Just like their human counterparts, the avian lymphocytes appear to function in the same manner with the B-cells (bursa dependent) having immunoglobulin receptors for antigens, while T-cells (thymus dependent) involved in cell mediated immunity (Campbell and Dein, 1984; Dieterlen-Lievre, 1988).

Reactive lymphocytes are small to medium sized with densely clumped chromatin and intensely basophilic cytoplasm. Nucleoli are usually absent, and a pale Golgi zone and vacuolation may be present. Reactive lymphocytes are usually seen in small numbers in

peripheral blood smears of healthy birds (Mitchell and Johns, 2008). However, an increased presence of reactive lymphocytes is commonly observed in birds that have infectious disease (Mitchell and Johns, 2008).

Blast transformed lymphocytes which are large lymphocytes with smooth dispersed chromatin may occasionally be observed in avian blood smears. There is abundant blue cytoplasm, and there is often a prominent Golgi zone, these cells are neoplastic, indicating lymphoid leukaemia or a leukaemic phase of lymphoma, but can also be seen as a result of immunologic stimulation (Mitchell and Johns, 2008).

Lymphocytosis usually occurs as a result of antigenic stimulation. This can occur in psittacine birds with viral disease, such as herpes virus or psittacine circovirus (Fudge and Joseph, 2000). This result is inconsistent as the same viral diseases may instead result in heterophilia or leukaemia (Fudge and Joseph, 2000; Schoemaker *et al.,* 2000). Lymphocytosis also occurs with wound healing, inflammatory diseases, parasitic infections, and viral diseases (Campbell, 2004a), and with lymphoid leukaemia (Latimer, 1994). Anaemia and thrombocytopaenia may also be present in birds with lymphocytic leukaemia (Latimer, 1994). Lymphocytic leukaemias are rare in birds in comparison to lymphosarcoma (Newell *et al.,* 1991).

2.11.2.6 Thrombocytes

Chicken and turkey thrombocytes are round to slightly oval cells with a round nucleus in the centre of a clear cytoplasm. Thrombocytes can be confused with small lymphocytes in tissue samples and blood smear. Monoclonal antibodies against chicken thrombocytes have been developed and can aid in identification (Horiuchi *et al.,* 2004). Avian thrombocytes contain azurophilic cytoplasm, granules, stain periodic acid Schiff (PAS) and Grimelius positive

clump readily and produce little thromboplastin. It is likely they are capable of phagocytosis, although definitive proof has not been obtained (Meseguer *et al.*, 2002).

2.12 Plasma or Serum Biochemistry

Serum chemistry and enzyme activities are valuable tools for monitoring the health and nutritional status of animals (Lumeij, 1997). Whole blood, plasma, and serum evalutions have been used extensively as measures of nutritional and metabolic status. The importance of established reference for biochemical parameters of free ranging birds is not only paramount for veterinarians dealing with captive and rehabilitating animals, but also for studying animals in their natural habitats. Recently, few studies have attempted to establish reference values of biochemical parameters for some birds (Ferrer, 1994; Balbontin and Ferrer, 2002; Casado *et al*., 2002). The gender, age, nutritional status, physiologic status and environment of the birds have great influence on the establishment of normal reference values for a given species of birds (Rodgers and Gass, 1983; Kaneko, 1997; Campbell, 1998; Bowerman *et al*., 2000; Artacho *et al*., 2007; Zaahkouk *et al.,* 2013*).* Also, it is often cumbersome to compare results from one laboratory with those obtained from another laboratory due to variations in methodology.

2.12.1 Total protein

Total protein level in birds is an important indicator of the health status and production features of the organism because of its numerous roles in the physiology. In veterinary clinical laboratories, it is the most frequently examined blood parameters for diagnostic purposes (Georgieva *et al.,* 2009). Refractometry of plasma gives consistently higher values when compared to the Biuret method in both plasma and serum which indicates that substances other than protein substantially add to the refractive index (Lumeji, 1987). In sick birds where only little amounts of blood can be collected, it may be advantageous to

determine total protein in plasma instead of serum (Hochleithner, 1994). The concentration of total protein in plasma is about 1.5 g/dl higher than serum because the former includes fibrinogen (Lumeji, 1987; Hochleithner, 1994). The total protein concentration by Biuret method range between 2.5 and 4.5 g/dl in normal birds (Campbell, 1988). Campbell and Dein (1984) reported that the total plasma protein values lower than 3.0 g/dl are hypoproteinaemic and birds with values less than 2.5 g/dl have poor prognosis. Amand (1985) reported that birds with total proteins less than 3.5 g/dl appear to have less of a chance to recover from their illness when compared to patients with values in the normal range. Moreover, these statements are invalidated by the fact that reference values for total serum protein in a number of healthy bird species are lower the above stated values (Lumeij, 1987). Although, determination of plasma proteins seldom leads to a specific diagnosis, it will help clinicians to evaluate the severity and progress of disease (Lumeji and Westerhof, 1987).

2.12.2 Calcium

Calcium plays two important physiological roles in the avian subject. First, it provides the structural strength of the avian skeleton by the formation of calcium salts. Second, it plays vital roles in many of the biochemical reactions within the body via its concentration in the extracellular fluid (Dacke, 2000). The control of calcium metabolism in birds has developed into a highly efficient homeostatic system, able to quickly respond to increased demands for calcium required for both their ability to produce megalecithal eggs and their rapid growth rate when young (Bentley, 1998; Dacke, 2000).

There are distinct differences between the mammalian and avian systemic regulations of calcium. The most dramatic difference between the two phyletic groups is in the rate of skeletal metabolism at times of demand. The domestic chicken will respond to hypocalcemic challenges within minutes compared with response to similar challenges in mammals that can

take over 24 hours (Koch, 1984). This is best demonstrated by an egg-laying bird where 10 % of the total body calcium reserves can be required for egg production in a 24-hour period (Klassing, 1998). This calcium required for eggshell production is mainly obtained from increased intestinal absorption and a highly labile reservoir found in the medullary bone, normally visible radiographically in female birds. The homeostatic control of the medullary bone involves oestrogen activity (Bentley, 1998).

2.12.3 Parathyroid hormone (PTH)

The parathyroid glands produce PTH from the chief cells in response to a low ionized calcium level (Hollis *et al*., 1997, Klassing, 1998). The hormone raises circulating Ca in three ways. Firstly, PTH increases mobilization of Ca from the skeleton by activating osteocytes to destroy bone matrix which releases trapped Ca while also stimulating osteoclasts to resorb bone tissue. Secondly, PTH promotes the absorption of Ca from the digestive tract by up-regulating the production of 1, 25-$(OH)_2$ cholecalciferol (1,25-$(OH)_2D_3$), the hormonal form of vitamin D_3. Finally, PTH stimulates the kidneys to excrete P while retaining Ca through re-absorption (Frandson and Spurgeon, 1992).

As in mammals, PTH has an essentially hypercalcemic action in birds, and if a parathyroidectomy is performed in quail, the birds respond with severe hypocalcemia unless calcium is given in the diet (Dacke, 2000). Birds appear more sensitive to PTH than mammals, reacting to intravenous injections of the hormone within minutes with a rise in blood calcium levels (Koch, 1984; Bentley, 1998; Dacke, 2000). The blood ionized calcium level feeds back on the chief cells in the parathyroid gland to tightly control secretion of the hormone. The hormone uses the kidney and the bone as its main target organs in birds (Dacke, 2000). In the bone, the hormone has rapid effects measurable within 8 minutes of administration compared with hours in mammals, so PTH is probably at least partially

responsible for the speed of calcium metabolism in the bird (Bentley, 1998). PTH directly stimulates osteoclasts to resorb bone. The hormone also will actively stimulate osteoblast activity, and it is thought that PTH-stimulated osteoblasts regulate osteoclast activity, providing the precise control system necessary in avian skeletal turnover (Bentley, 1998; Dacke, 2000). PTH binds to osteoclasts, increasing bone resorption by stimulating their metabolic activity and division. PTH also has direct influence on both calcium and phosphorus excretion in the bird. Calcium excretion is increased and phosphorus decreased following parathyroidectomy. These changes can be reversed by injections of PTH.

2.12.4 Oestrogen

Oestrogen is a steroid hormone produced by the developing follicles in the ovaries. It is one of the primary steroid hormones which are lipophilic and poorly soluble in water (Shore and Shemesh, 2003). The most common oestrogens are oestrone, oestrodiol-17 and oestroil. Oestrone and oestrodiol- 17 are the most important steroids because they are physiologically effective at lower concentrations than other steroids found in the environment. It is speculated that reproductive hormones can improve egg laying, performance in laying birds especially at old age, when the adult laying birds decline in production and also the shell quality deteriorate (Hansen *et al*., 2003).

In birds, oestrogen is the differentiating hormone for gonadal development, its presence in the female is the differentiation antigen for the heterogametic gonad in the avian ovary. When oestrogen is absent, the gonad develops into testes, but when present for the normal female development the left gonad develop into an ovary while the right gonad regresses (Giesy *et al*., 2003). Oestradiol plays a central role in the determination of egg mass and quality. It promotes the formation of the vitellogenins from the liver. These are lipoproteins that are incorporated into the egg yolk. They bind calcium, and their production is followed by a rise

in serum calcium levels. This oestrogen-controlled hypercalcemic effect is not seen in mammals and is thought to be due to the need to produce large calcified eggs, requiring a rapidly mobilized source of calcium. Oestrogens also influence the mobilization of medullary bone during the egg-laying cycle (and also during the nocturnal fast). The effect of oestrogen on avian medullary bone is a large research area due to the importance of oestrogen in maintaining bone mass in postmenopausal women (Bentley, 1998; Dacke, 2000). Bentley, (1998) reported that oestrogen increases the serum growth hormone receptors in the kidneys and directly reverses the renal activity of hydroxylase which is responsible for the formation of 1,25 dihdroxycholecalciferol that mediates in intestinal absorption of calcium thereby increasing the concentration of calcium binding protein in the target cells (Aslam *et al*., 1998). Hochleithner, (1989) observed that oestrogen implants improve calcium uptake throughout the intestine and that there is a strong interrelationship between the weight of egg and shell thickness with the rate of calcium absorption and availability. This could be the reason for the observed effects of oestrogen on shell thickness.

The particular protein concerned in the binding of the increased plasma calcium is the phosphorus-containing protein phosvitin. It is the characteristic protein of the egg yolk. Phosvitin has a great affinity for calcium, the greater the amount of this phosphoprotein in the blood, the higher the level of plasma calcium. Phosvitin is synthesized in the liver under the influence of oestrogen and is carried in the blood (in combination with lipid material) to the follicles developing in the ovary. Similar proteins are found in the blood of all animals that lay yolky eggs, including fishes, amphibians and reptiles, and yet neither fishes nor amphibians lay eggs with calcified shells, and among the reptiles only the Chelonia (turtles and tortoises) and the Crocodilia (Taylor, 1970).

As hens mature sexually, oestrogen concentrations in plasma gradually increase, with more marked increases occurring from16 to 20wk of age (Johnson and van Tienhoven, 1980; Whitehead and Fleming, 2000; Madison *et al.*, 2002). Concentrations remain high for the next several weeks with daily surges occurring approximately 4 to 6 hours prior to ovulation and coincident with the daily surges of luteinizing hormone (LH) and progesterone (Johnson and van Tienhoven, 1980; Etches, 1987). The exact time course of the secretion pattern of oestrogen after hens reach peak production is not clear; few studies have monitored plasma oestrogen systematically over the entire production period, and those that have done so have not taken samples often enough to give a clear picture. It is generally accepted that oestrogen declines over the production year (Johnson, 1986), drops very low during molt (Hoshino *et al*., 1988), and increases again as hens are brought back into production (Johnson, 1986; Etches, 1987). These changes underlie the egg production patterns of commercial layers, in which there is a gradual decline in egg numbers from the peak reached shortly after sexual maturity. When commercial layers drop below 80 % production, 3 molt regimens are initiated that take hens out of production from 68 to 72 week of age. This induces a rest of the reproductive system and a return to reproductive condition for a second laying cycle. In a recent study conducted to establish more definitively oestrogen-Ca relational changes over time in the hen, Hansen *et al*. (2003) confirmed the dramatic decrease in oestrogen in hens at 70 weeks compared to those in peak production (29 weeks). Beck and Hansen (2004) also documented parallel changes in oestrogen receptor populations in both kidney and duodenum. As shown by immunochemistry staining and Western blot analysis, ERα decreased with age in hens (Hansen *et al*., 2003); Ca absorption by duodenal tissue also decreased linearly from prelay highs, which agrees with earlier work on Ca absorption and CaBP-D28K concentrations (Berry and Brake, 1991; Al-Batshan *et al*., 1994).

2.12.5 Progesterone

Progesterone is the starting compound in the formation of other sex hormones, both male and female (Gryzińska *et al.,* 2011). This hormone is produced in the ovary by the corpus luteum, and in animals by the attaching placenta, where it is responsible for maintaining pregnancy. In birds, due to the different manner of bringing offspring into the world, the corpus luteum does not arise. Progesterone was isolated in birds to the left gonad (Sturkie, 1970). The right gonad disappears during the embryonic stage and in adult females, it only acts in the rudimentary form, which is visible only microscopically (Turner and Bagnara, 1978). Once follicles start to grow they are either selected for ovulation or, like the majority of them, they become atretic. The underlying molecular mechanism for follicle degeneration is apoptosis (or programmed cell death) as shown for all vertebrate species analysed so far (Tilly *et al.,* 1997) and even for invertebrates (Criel and D'Herde, 1996). Follicular atresia is initiated by programmed cell death of the somatic cells surrounding the oocyte: the granulosa cells (Tilly *et al.,* 1997). Several studies suggest that P4, the major steroidal output of the preovulatory granulosa, can regulate granulosa cell proliferation (Chaffkin *et al.,* 1992) and inhibit apoptosis in granulose cultures (Luciano *et al.,* 1994). Progesterone affects nerve centres via the hypothalamic area in the secretion of pituitary gonadotrophic hormones and the nervous systems condition. This relationship also exists between the male reproductive system and pituitary system. Evidence supporting this fact can be obtained by removing the pituitary gland which causes symptoms similar to those of castration, and those that occur after excision of the gonads. Progesterone moving via different neural mechanisms regulates the release of the hormone LH, and thus begins the process of ovulation, occurring a few minutes after the deposit of eggs (Sturkie, 1970).

In the body of mammals, progesterone along with oestrogen stimulates the glands and secretory cells lining the fallopian tubes and uterus, preparing for the latter implantation of the embryo. In birds while in the oviduct, progesterone induces the production of the protein avidin. This is a biotin-binding protein in egg white having a very high affinity for biotin (Green, 1975). Both oestrogen and progesterone are necessary for normal reproductive cycle of birds (Turner and Bagnara, 1978). From *in vitro* incubation studies, it has been clarified that the primary source of P4 in avian species is the granulosa cell of the largest (F1) follicle, the one destined to ovulate next. In the smaller pre-ovulatory follicles, the second (F2) and the third (F3) largest follicles, P4 is rapidly metabolized due to their significant higher amounts of conversion enzymes in the theca cells (Huang and Nalbandov, 1979, Mori *et al.*, 1984; 1985; Mori, 1987).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Location of Experimental Study

This study was carried out in the Department of Veterinary Pathology, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria, Kaduna State, which is located within the Northern Guinea Savannah Zone of Nigeria, between latitude 7^0 and 11^0N, and longitude 7^0 and 44^0E. The average rainfall of this zone ranges from 1,000 to 1,250 mm, and the average temperature ranges from 19^0C to 33^0C (Sawa and Buhari, 2011).

3.2 Experimental Animals and Design

A total of twenty (20) laying (20 weeks old) chickens that were specifically raised for research purposes and vaccinated against endemic vaccinable diseases in the area, except *E. coli* infections, were purchased from a reputable farm in Kaduna State. The birds were housed and managed intensively in the poultry research pens of the Department. Prior to arrival of the birds, the pens were thoroughly washed with detergent and sprayed with formalin at a concentration of 4 ml/litre of water.Throughout the experiment, the birds were fed standard commercial layer mash (Hybrid Feeds®) and water was provided to the birds *ad libitum.*

3.2.1 The bacterial organism

The *E. coli* used in this experiment, APEC serotype O1K1, was obtained from the bacteria bank of the National Veterinary Research Institute, Vom, Plateau State, Nigeria.

3.2.2 Grouping and inoculation of birds with *E. coli*

The birds were kept for 4 weeks to acclimatize to the new environment and other handling conditions, after which they were divided at random into two groups (infected and control) of

10 layers each. The control birds were housed in a pen located far away from the pen in which birds of the infected group were housed. The bacteria from a previously prepared slant were reactivated by sub-culturing on Eosin and methyline blue (EMB). The resulting colonies were then examined for their characteristic features, colour, morphology and tested for gram stain reaction. On the day of infection (Day 0), bacterial inoculum was prepared using McFarland standards, which were prepared by adding barium chloride to sulphuric acid to obtain a barium precipitate of different turbidity standards. These were used to estimate the number of bacteria present in a liquid suspension (McFarland, 1907). In this test, the turbidity of suspension of bacteria was compared with turbidity of the appropriate standard. Each of the birds in the infected group was then challenged by inoculating each of the birds in the infected group with 0.5 ml of bacterial aliquot containing 10^9 colony forming units (CFU) of the bacteria intratracheally (Antao *et al*., 2008). After inoculation, the bacteria were recovered from infected birds by following conventional culture, isolation and identification of bacteria by standard procedures as documented in Cowan and Steel (Barrow and Feltham, 2000) and Cheesbrough (2006).

3.3 Clinical Observations

Starting from day 0 of infection and throughout the experimental period, birds in the infected group were closely monitored for clinical signs of colibacillosis and recorded accordingly. Five birds, each, from the infected and control groups were selected at random and their live body weights were determined on each day of blood sampling. Also, egg production was monitored and the weekly percentage egg production of birds in the infected and control groups was calculated.

3.4 Collection of Blood Sample for Haematological, Reproductive Endocrine and Biochemical Analyses

Beginning from day 0 and, subsequently, on days 2, 4, 6, 14, 21, 28, 35 and 42 post-infection, blood samples (3 ml) were collected from the brachial vein of each of 5 birds selected at random from each group, at 08:00 to 09:00 hours of the day, using 23 G needles. Exactly 2 ml of the blood was dispensed into heparinized vacutainer tube. The blood was centrifuged in the laboratory at 2200 *g* for 10 minutes. The plasma was then dispensed into vials and stored at -20 ^{o}C until used for determination of activities of parathormone, oestrogen, progesterone, AST and ALT and calcium and phosphate concentrations. The remaining 1 ml was dispensed into EDTA-coated vacutainer tube and used for haematological evaluations.

3.5 Haematological Evaluations

3.5.1 Determination of packed cell volume

Packed cell volume (PCV) was determined using the microhaematocrit method (Feldmann *et al*. 2000). Microhaematocrit capillary tubes were filled with blood by capillary action to about 75% of their lengths, and outside of the tubes were carefully dried with cotton wool after filling each of the tubes with blood.The opposite ends were then sealed with a flame from bunsen burner. The sealed tubes were then packed in a haematocrit centrifuge machine (to touch the rim). Blood samples were centrifuged in heparinised capillary tubes for 5 minutes at 4,383 xg using the Saitexiangyi TG12MX® Micro-haematocrit centrifuge machine. Then the proportion of cells in total volume of blood was measured and recorded as a percentage using the Hawskley® Microhaematocrit Reader.

3.5.2 Determination of haemoglobin concentration

Blood haemoglobin concentration was assayed colorimetrically using cyanmethhaemoglobin method described by Feldmann *et al.* (2000). Five millilitre of haemiglobincyanide (HICN)

(Drabkin) solution were measured using a 5 ml syringe into plastic test tubes. Twenty microliter (20 µl) of blood was measured using a micropipette and added to the Drabkin solution in the test tube and properly mixed by gently shaking the test tube. It was centrifuged at 1,509 x *g* for 15 minutes to separate the empty RBC from interfering with the reading. The supernatant was separated into a sample bottle. The mixture was absorbed into the haemoglobin meter (XF–C, China). After the wiggling pump stops working, the value displayed on the screen was recorded in g/dl as the haemoglobin concentration.

3.5.3 Determination of red blood cells and total white cell count

Red blood cells (RBC) count was determined with the NatHerrick solution (1:200 dilutions) and the Improved Neubauer haemocytometer (Campbell and Ellis, 2007). The heparinised blood samples were slightly agitated and the RBC diluting pipette was used to pipette the blood to the 0.5 mark. The tip of the pipette was cleaned properly using a tissue paper without touching the distal opening of the pipette tip with tissue, as this will cause capillary shift of blood into the tissue. The diluting solution (Natt-Herrick) was also pipette to the 101 mark (1:200) without entirely immersing the pipette tip into the diluting fluid. The mixture was well shaken to obtain sufficient dilution. Cover slip was apllied. The first 5 drops were discarded before filling the counting chambers of the improved Neubauer haemocytometer and the cells were allowed to settle.

The light microscope (Olympus-XSZ-107BN), at low power magnification (X40) was used to view the cells and counting was done using the tally counter.

For RBC count, the cells contained in the four corner and central squares in the mid-section of the haemocytometer were counted. Following the “L” rule: cells that touch the centre triple lines of the ruling on the left and the bottom sides were counted but cells that touch the centre

triple lines of the ruling on the right and the top sides were not counted. The RBC count was calculated using the formula below:

RBC count = N/5 x 25 x DF x 10 = N x 5 x 200 x 10 = N x 10000/µL

Where N = Total number of RBC counted in the 5 squares in the mid-section of the haemocytometer (or in160 squares).

The WBC in the four outer large squares of the haemocytometer were counted and calculated using the formula below:

$$N \times 500 = WBC \times 10^3 /\mu l \text{ or } WBC \times 10^3 \times 10^6/L$$

Where N = Number of WBC counted in the four outer large squares (or in 64 small squares)

Note that both charged sides of the haemocytometer were counted for both the RBC and TWBC and the average calculated.

3.5.4 Calculation of erythrocytic indices

The mean corpuscular volume (MCV) and mean corpuscular haemoglobin concentration (MCHC) were calculated using the following standard formulae (Campbell and Ellis, 2007):

MCV = (PCVx10)/RBC =MCV (fl)

MCHC = (Hb x 100)/PCV=MCHC (g/l)

3.5.5 Determination of differential white blood cell count

A small drop of blood (about 2 µl) was used for the preparation of blood smears each using the standard slide-to-slide technique. The air-dried smears were properly labelled using pencil on the frosted end of the slide and then fixed in a fixing jar containing methanol for 3 minutes and air-dried. Staining was done by flooding the smears with Wright-Giemsa stain for 3

minutes. An equal amount of Sørensen's buffer (pH of 6.8) was added and then mixed gently by blowing using a pipette until green metallic sheen forms on the surface. This was allowed to stand for further 6 minutes. The smears were rinsed with the Sørensen's buffer and allowed to stand for a minute for differentiation. The stained slides were then washed copiously with Sørensen's buffer and the back of the smears were wiped with tissue paper to remove the excess stain and allowed to air dry. Examination of the blood smears was done using light microscope (Olympus-XSZ-107BN) under high-power magnification with oil immersion (X1000). One hundred WBC were counted and classified based on their morphologic features (Campbell, 1988; Hawkey and Dennet, 1989; Campbell and Ellis, 2007). The counting was done using Marble® Blood Cell Calculator. The differential WBC count was then expressed as apercentage of the individual cell group.

The percentage of each cell was then converted into absolute numbers by reference to the total WBC using the formula below:

$$\frac{\text{Percentage of WBC counted x TWBC}}{100} = \text{Absolute Number x } 10^9 /l$$

3.6 Determination of Progesterone, Oestrogen and Parathormone Profiles Using ELISA Kits

Plasma progesterone, oestrogen and parathormone profiles were measured using commercial ELISA kits (Monobind Inc., USA, Accubind progesterone, oestradiol and parathormone ELISA microwells), in accordance with the manufacturer instructions.

3.6.1 Progesterone determination

Exactly 25 µl, each, of plasma reference calibrator, control and specimen was pipetted into an assigned well. Progesterone Enzyme Reagent (50 µl) was then added to all wells. The microplate was then gently swirled for 20 seconds to mix. Progesterone Biotin Reagent (50 µl) was then added to all wells. Again, the microplate was swirled for 20 seconds. The

microplate was then covered and incubated for 60 minutes at room temperature. The content of the microplate was then discarded by decantation. The plate was blot dried with absorbent paper. Exactly 350 µl of wash buffer was add to each well, and then decanted. This procedure was repeated twice. Substrate solution (10µl) was then added to all wells. Reagents were added in the same order to minimize reaction time differences between wells. The plate was then incubated for 20 minutes at room temperature. Exactly 50 µl of stop solution was added to each well and then gently mixed for 20 seconds. Absorbance in each well was read at 450 nm in a microplate reader (BIO-TEK Instruments Inc., USA). The results were read within 15 minutes after adding the stop solution.

3.6.2 Oestrogen determination

Exactly 25 µl, each of the plasma reference calibrator, control or specimen was pipetted into an assigned well. Biotin Reagent (50 µl) was added to each well. Exactly 50 µl of the conjugate Reagent was added to each well. The microplate was then incubated for 90 minutes at room temperature on a plate shaker. The content of the microplate was then discarded by decantation. Exactly 300 µl of wash buffer was added to each well and then decanted. This procedure was repeated three times for a total of 4 washes. Exactly 100 µl of TMB substrate was added to all wells. The plate was then incubated for 15 minutes at room temperature. Exactly 50 µl of Stop Solution was added to each well and mixed gently for 15 seconds. The absorbance in each well was read at 450 nm in a microplate reader (BIO-TEK Instruments Inc., USA). The result was read within 15 minutes after adding the stop solution.

3.6.3 Parathormone determination

Exactly 25 µl of the appropriate plasma reference calibrator, control or specimen was pipetted into an assigned well. Biotin Reagent (50 µl) was added to each well. Exactly 50 µl of the conjugate Reagent was added to each well. The content of the microplate was incubated for

90 minutes at room temperature on a plate shaker. The content of the microplate was discarded by decantation. Exactly 300µl of wash buffer was added and then decanted. TMB substrate (100µl) was added to all wells. The plate was then incubated for 15 minutes at room temperature. Exactly 50 µl of stop solution was added to each well and mixed gently for 15-20 seconds. The absorbance was read in each well at 450 nm with microplate reader (BIO-TEK Instruments Inc., USA). The result was read within 15 minutes after adding stop solution.

3.7 Determination of Plasma Calcium and Phosphate Concentrations

Calcium and phosphorus concentrations were determined measured using commercial test kits (Agappe, India) by means of an ultraviolet digital spectrophotometer (Perkin Elmer AAS 400).

3.7.1 Measurement of plasma calcium ion (Ca^{2+}) concentration

The principle is based on the reaction of calcium ions (Ca 2++) with O-cresolphthalein complex in an alkaline solution to form an intense violet coloured complex which shows maximum absorbance at 578nm. The 8-hydroxy quinoloine prevents Mg2++ interference upto 4 mmol/L.

Reagent composition:

- Calcium dye reagent (R2): Diethylamine (360 mmol/L)
- Calcium base reagent (R1): O-Cresolphthalein complex (0.15 mmol/L) and 8-Hydroxyquinoline (17.2 mmol/L).
- Calcium standard: Calcium standard concentration 10 mg/dL

Sample: plasma (free of haemolysis)

General system parameter

- Mode of Reaction End point

- Slope of reaction Increasing
- Wavelength 578 nm (565 -580nm)
- Temperature $30^{o}C$
- Standard Concentration 10 mg/dL
- Linearity 15 mg/dL
- Blank Reagent
- Incubation time 5 min
- Sample volume 10 µL
- Reagent volume 1000 µL
- Cuvette 1 cm light path

Laboratory procedure:

	Blank	Standard	Sample
Working Reagent	1000 µL	1000 µL	1000 µL
Standard	-	10 µL	-
Sample	-	-	10 µL

The sample and standard were mixed gently and incubated for 5 minute at room temperature

The absorbance of standard and sample were measured against the reagent blank at 546 nm

Calculation:

$$\text{Calcium Con. (mg/dL)} = \frac{\text{Absorbance of Sample}}{\text{Absorbance of Standard}} \times 10$$

3.7.2 Measurement of plasma phosphorus (P) concentration

The principle is based on determination of inorganic phosphorous according to the following reaction:

Phosphorous

Ammonium molybdate + sulphuric acid ---------->phosphomolybdic complex

Reagent composition:

- Inorganic phosphorus reagent : Sulphuric acid (210 mmol/L) and Ammonium molybdate (650 mmol/L)
- Inorganic phosphorus standard: Inorganic phosphorus standard concentration 5 mg/dL

Sample: plasma (free of haemolysis)

General system parameter:

- Mode of Reaction End point
- Slope of reaction Increasing
- Wavelength 340 nm
- Temperature 37^{o}C
- Standard Concentration 5 mg/dL
- Linearity 15 mg/dL
- Blank Reagent
- Incubation time 1 min
- Sample volume 20 µL
- Reagent volume 1000 µL
- Cuvette 1 cm light path

Laboratory procedure:

	Blank	Standard	Sample
Reagent	1000 µL	1000 µL	1000 µL
Standard	-	20 µL	-
Sample	-	-	20 µL

The sample and standard were mixed gently and incubated for 1 minute at room temperature

The absorbance of standard and sample were measured against the reagent blank at 505 nm

Calculation:

$$\text{Phosphorus Con. (mg/dL)} = \frac{\text{Absorbance of Sample}}{\text{Absorbance of Standard}} \times 5$$

3.8 Determination of Liver Enzymes

AST and ALT activities were determined by the enzymatic method using kits (Agappe Diagnostic laboratory, Switzerland) in accordance with the manufacturer instructions.

3.8.1 Determination of AST

The principle is based on kinetic determination of AST according to the following reaction:

$$\text{L-Aspartate + a-ketoglutarate} \xrightarrow{\text{AST}} \text{oxaloacetate + L-Glutamate}$$

$$\text{Oxaloacetate + NADH + H}^{-} \xrightarrow{\text{LDH}} \text{L-Malate + NAD}^{-}$$

AST = Aspartate amnotransferase

LDH = Lactate dehydrogenase

Exactly 1000 µl of the working reagent was mixed with 100 µL of the sample. The mixture was incubated at 37^0 C for 1 minute. The change was measured in absorbance per minutes (ΔOD/min) within 3 minutes. The AST activity (U/L) was calculated using the formula below:

AST activity (U/L) = ΔOD/min x 1745.

3.8.2 Determination of ALT.

The principle is based on kinetic determination of ALT according to the following reaction:

$$\text{L-Alanine + alpha-ketoglutarate} \xrightarrow{\text{ALT}} \text{Pyruvate + L-Glutamate}$$

$$\text{Pyruvate + NADH + H}^{-} \xrightarrow{\text{LDH}} \text{L-Lactate + NAD}^{-}$$

ALT = Alanine amnotransferase

LDH = Lactate dehydrogenase

Exactly 1000 µl of the working reagent was mixed with 100 µL of the sample. The mixture was incubated at 37^0 C for 1 minute. The change was measured in absorbance per minutes (ΔOD/min) within 3 minutes. The ALT activity (U/L) was calculated using the formula below:

ALT activity (U/L) = ΔOD/min x 1745.

3.9 Determination of Total Plasma Protein Concentration

Total plasma protein was estimated using hand refractometric method (Sirois, 1995). After reading the PCV, the haematocrit capillary tube was broken at the point immediately above the buffy coat. The end containing the buffy coat and packed red cells was discarded retaining the end containing the plasma. The plastic flap of the hand refractometer was reflected to expose "glass'' sample surface which was thoroughly wiped with cotton wool; the broken end of the capillary tubes was then used to make contact with the sample surface of the refractometer in order to dispense plasma sample unto the surface thereafter pressure was then applied on the flap against the plasma sample with the index finger and holding the refractometer against light. The concentration of plasma proteins was then read in grams/deciliter (g/dl).

3.10 Postmortem Examination

Birds that died during the course of the experiment were subjected to post-mortem examination; gross lesions observed were recorded accordingly. The specimens from the liver, ovarian follicles and other organs were collected into bottles containing 10% neutral buffered formalin and subsequently processed, using standard techniques, for histopathological examination.

Birds that survived up to the end of the experiment but showing clinical signs of colibacillosis were humanely euthanized by cervical dislocation, after collecting the last set of blood samples. The birds were necropsied and specimens were similarly collected and used for histopathological examination, as previously described.

3.11 Statistical Analysis

All the data obtained were subjected to statistical analysis including the calculation of the means and standard error of the means. Data between groups were evaluated using student t-test and values of $P < 0.05$ were considered significant using Graph Pad prism version 5.00 for windows, Graph pad Software, San Diego California USA.

3.12 Ethical Approval

Ethical approval was sought for from the Ethical Committee on Animal Use and Care of Ahmadu Bello University, Zaria (ABUECAUC), with Approval number: ABUECAUC/2017/026

CHAPTER FOUR

4.0 RESULTS

4.1 Clinical Observations on the Infected Layers

Following infection with APEC serotype O1K1, birds in the infected group appeared clinically normal until day 3 pi, when the birds started to show reduction in feed and water consumption, yellowish watery faeces (Plate I) and weakness. By day 4 pi, loss of weight in this group of birds was evident. During the experiment, two birds in the infected group died of the disease, one on day 10 and the other on day 35 pi, representing 20 % mortality. All the birds in the control group were apparently healthy up to termination of the experiment.

Plate I: ***E. coli*-infected birds showing soiled vent from diarrhea**

4.2 *E. coli* Recovery

Escherichia coli organisms were recovered from the blood of the infected birds showing clinical signs of colibacillosis as indicated by presence of colonies with green metallic sheen on the EMB culture (Plate II). To further confirm the bacterium, Gram's staining was carried out and the organism was a Gram negative rod which was catalase positive, produced acid and gas on triple sugar iron agar. It was indole positive, citrate and urease negative, methyl red positive, Voges Proskauer negative and was motile.

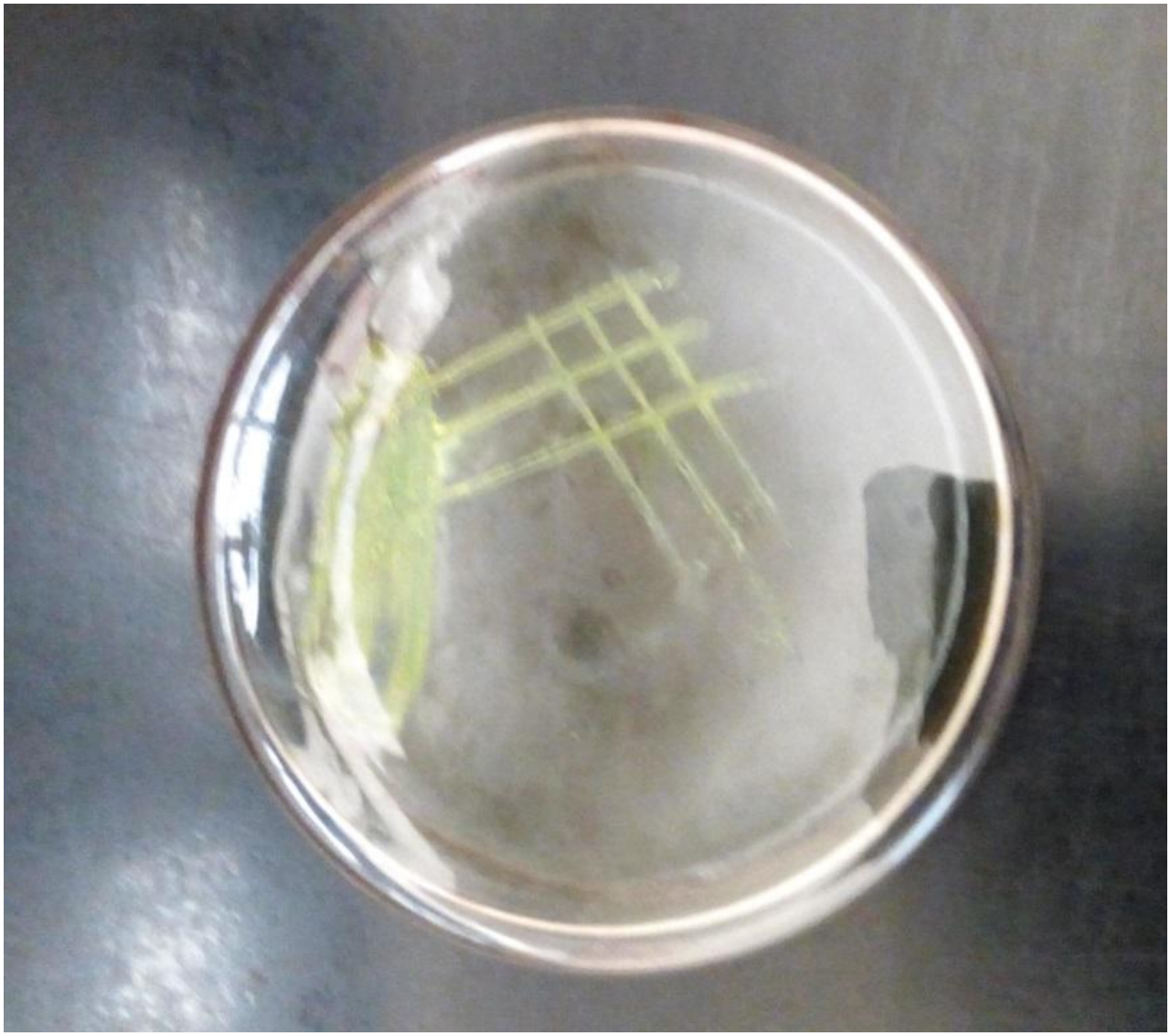

Plate II: *E. coli* culture on EMB showing characteristic green metallic sheen recovered from blood of bird in the infected group.

4.3 Body Weights

The mean body weights of birds in the infected and control groups were as shown in Figure 4.1 and Apprendix 1. The daily mean weight of *E. coli*-infected group of layers showed a significant decrease ($P<0.05$) that was progressive starting from day 4 pi value (1576 ± 44.56 g) to a lowest value (1480 ± 48.89 g) on day 6 pi followed by a progressive but non-significant ($P>0.05$) increase in the infected layers, beginning from day 28 and continued until termination of the experiment. On the contrary, an increase in mean body weight was observed in the control group, all through the study.

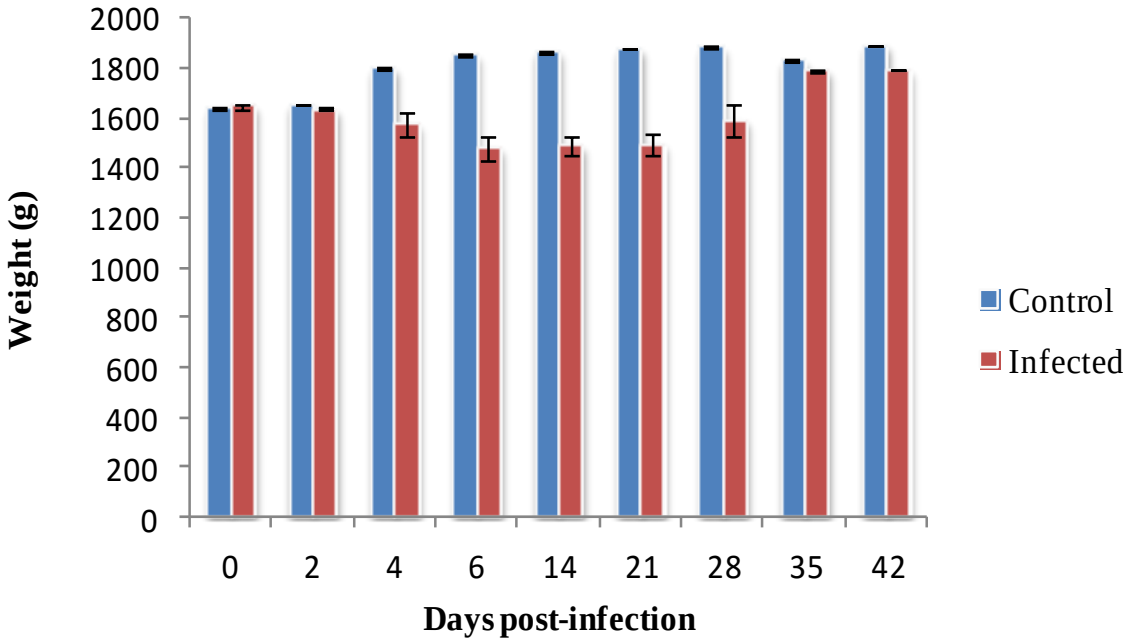

Figure 4.1: Mean (± SEM) daily weight in *E. coli*-infected and control layers

4.4. Effect of *E. coli* Infection on Egg Production in Layers.

4.4.1 Weekly percentage egg production

The percentage egg production in the infected and control groups are shown in Figure 4.2 and Appendix 2. A significant ($P < 0.05$) progressive decrease in the mean weekly percentage egg production was observed in the *E.* coli-infected layers starting from week 1 to reach the lowest value of 40 ± 4.47% in week 3. Following this, the percentage egg production remained consistently low with minor fluctuations until termination of the experiment at week 6.

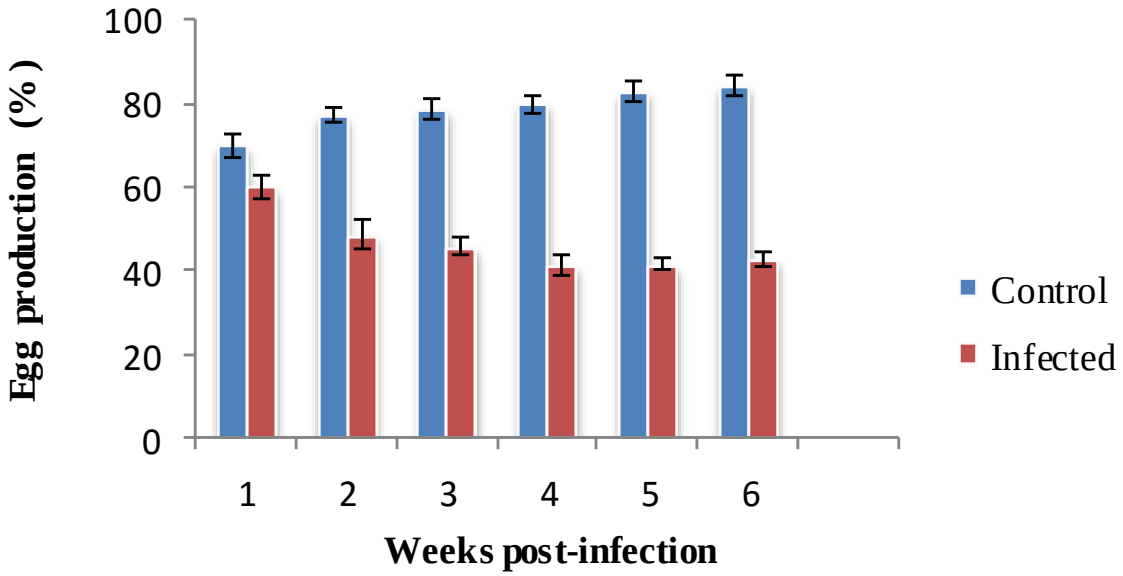

Figure 4.2: Percentage egg production in the *E. coli*-infected and control layers

4.5. Effect of *E. coli* Infection on the Haematological Values of Layers

4.5.1 Packed cell volume (PCV)

The mean PCV in the *E. coli*-infected and control layers are presented in Figure 4.3 and Appendix 3. A progressive and significant ($p< 0.05$) decrease in the mean PCV was observed in the infected group from day 2 to a lowest value (17 ± 0.71%) on day 4 pi. Thereafter, the mean PCV in the infected group gradually rose to its peak level (24.6±1.03%) on day 28 pi. Following this, the mean PCV stabilized, with non-significant fluctuations until termination of the experiment.

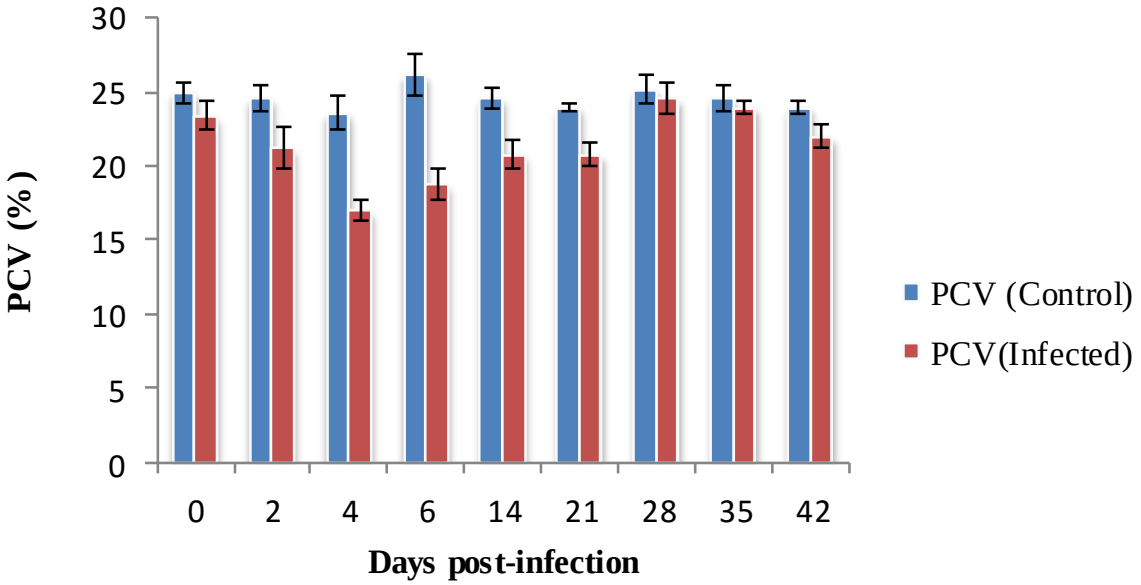

Figure 4.3: Mean (± SEM) Packed cell volume (PCV) in the *E. coli* - infected and control groups of layers

4.5.2 Red blood cell (RBC) counts

The mean RBC count in the *E. coli*-infected and control layers are presented in Figure 4.4 and Appendix 3. The mean red blood cell count (RBC count) steadily decreased beginning from day 2 pi (1.92 ± 0.04 x 10^{12}L) to the lowest value (1.63 ± 0.07 x 10^{12}L) on day 14 pi, that differed significantly ($p< 0.05$) from that (2.21 ± 0.04 x 10^{12}L) of the control group. A sharp increase was observed in the mean RBC count from day 21 (1.76 ± 0.12 10^{12}L) to the highest (2.55 ± 0.08 10^{12}L) on day 28 pi. Thereafter, the mean RBC count slightly decreased and stabilized until termination of the experiment. The mean RBC count in the control group remained fairly unchanged throughout the experiment.

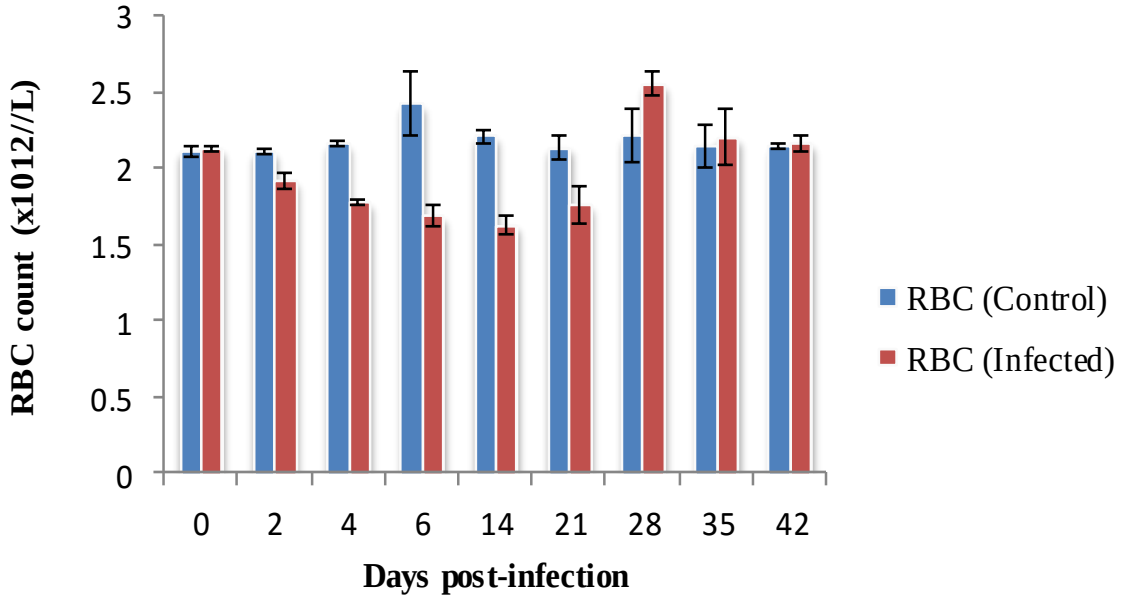

Figure 4.4: Mean (± SEM) red blood cell count in *E. coli*-infected and control groups in layers

4.5.3. Haemoglobin (Hb) concentration

The mean Hb concentration in the *E.* coli-infected and control layers is presented in Figure 4.5 and Appendix 3. The mean Hb concentration progressively decreased in the infected group beginning from day 2 pi (7.11 ± 0.47g/dl) to a lowest value (5.67 ± 0.24g/dl) on day 4 pi that differed significantly ($p<0.05$) from the corresponding value in the control group. Afterwards, it gradually increased to reach levels comparable with those of the control on days 28, 35 and 42 pi.

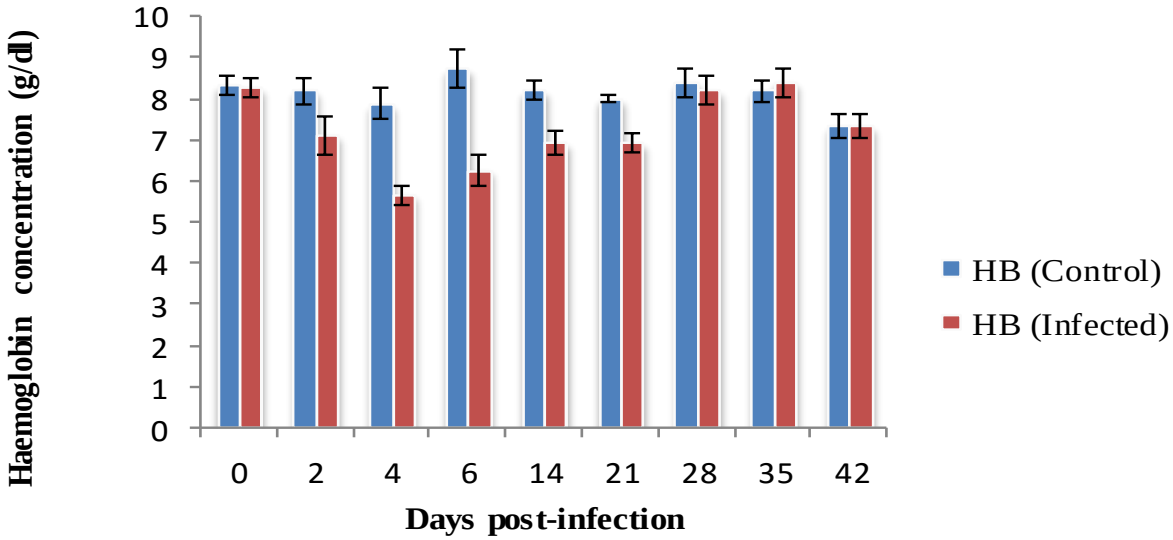

Figure 4.5: Mean (± SEM) Haemoglobin concentration in the *E. coli*-infected and control groups of layers

4. 5.4 Mean corpuscular volume (MCV)

The mean MCV of erythrocytes in the *E. coli*-infected and control layers is presented in Figure 4.6 and Appendix 3. Following the infection with *E.* coli, no significant ($p>0.05$) change in the mean MCV was observed until day 14 when it rose in the infected group to a significantly ($p<0.05$) higher value (127.54 ± 12.26 fl) than that of the control group. It then stabilized to levels comparable with those of the control up to termination of the experiment.

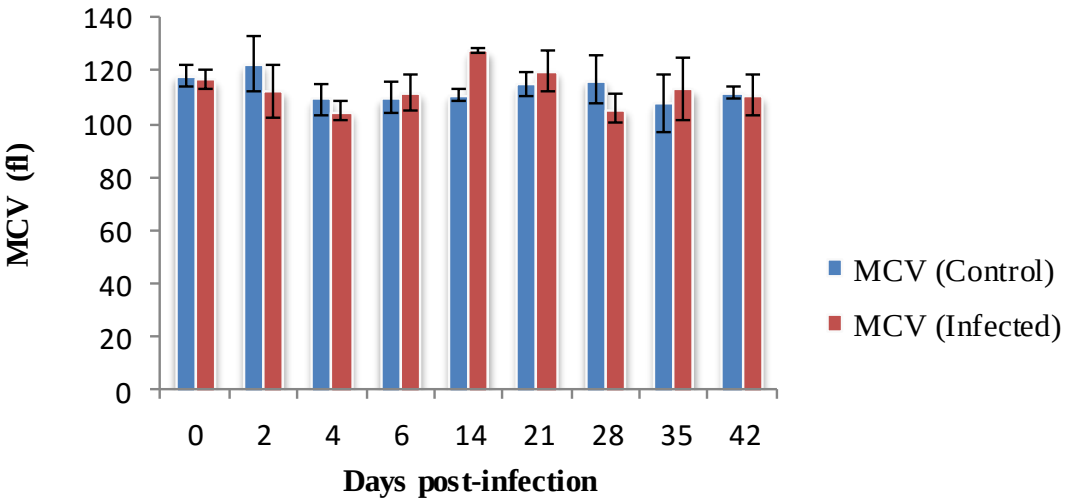

Figure 4.6: Mean (± SEM) corpuscular volume (MCV) of erythrocytes in the *E. coli*-infected and control groups of layers

4. 5.5 Mean corpuscular haemoglobin concentration (MCHC)

The mean MCHC of erythrocytes in the *E. coli*-infected and control layers is presented in Figure 4.7 and Appendix. The mean MCHC profile following the infection remained fairly unchanged and comparable with those of the control group, except for the non-significant (p>0.05) fluctuations throughout the experiment.

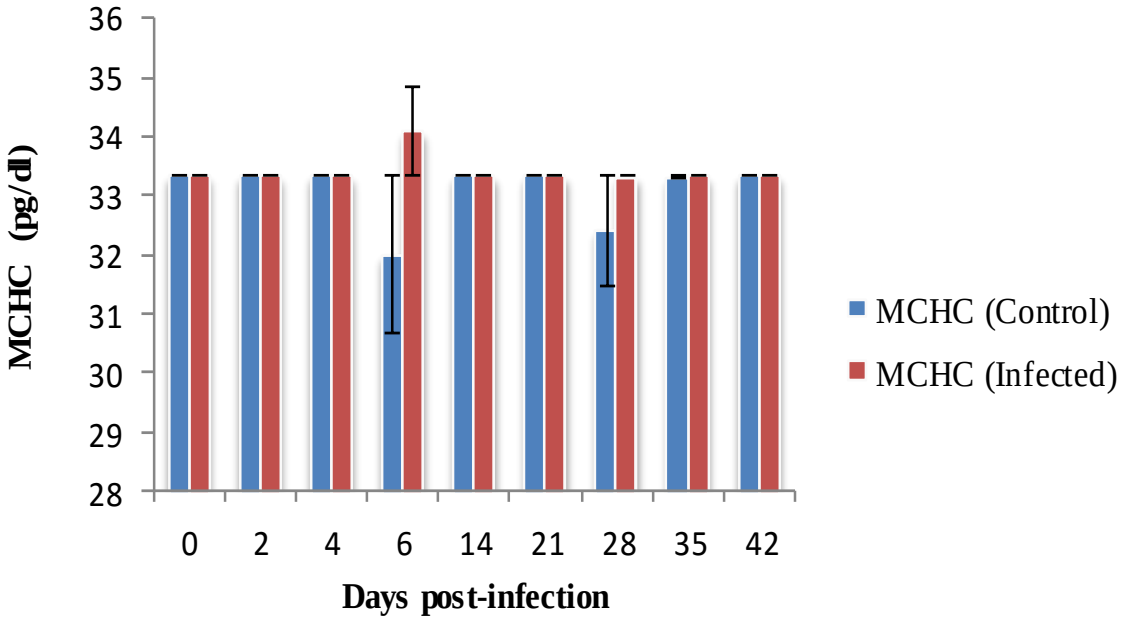

Figure 4.7: Mean (± SEM) Mean corpuscular haemoglobin concentration of erythrocytes in the *E. coli*-infected and control groups of layers

4. 5.6. Total white blood Cell (WBC) counts

The mean total WBC count in the *E. coli*-infected and control layers is presented in Figure 4.8 and Appendix 3. The mean total WBC count rapidly increased in the infected group from day 2 (14.81 ± 3.28x 10^9/L) to a peak level (29.16 ± 0.81 x 10^9/L) on day 14 pi. Following this, the mean value of this parameter in the *E. coli*-infected group showed progressive decline up to the end of the experiment. The values of the WBC count during the infection were significantly ($p< 0.05$) higher in the infected than that in the control group.

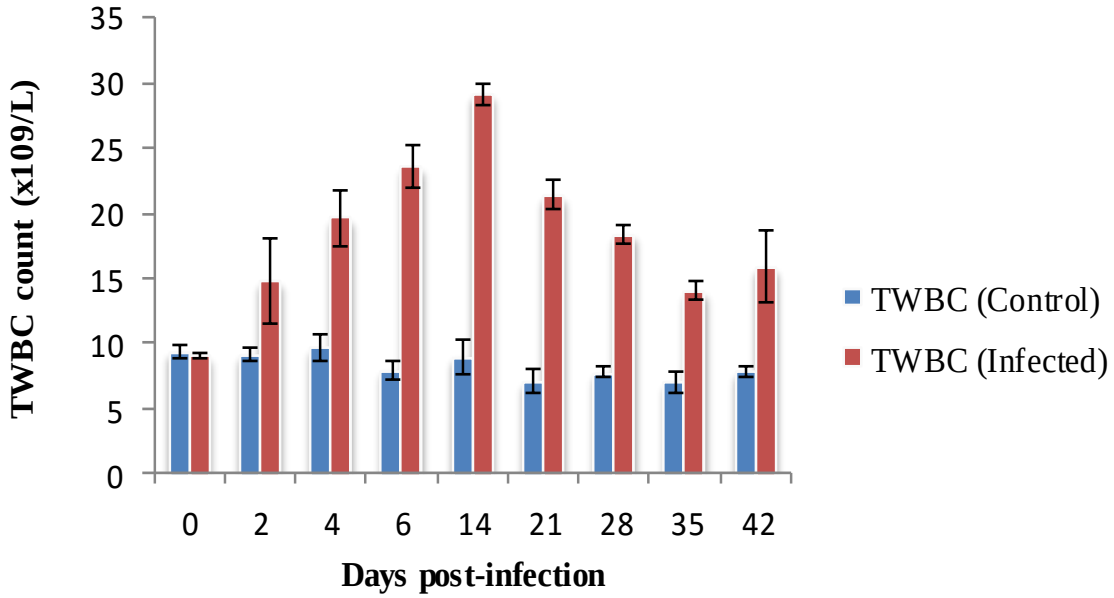

Figure 4.8: Mean (± SEM) total white blood cell count in the *E. coli*-infected and control groups of layers

4. 5.7 Heterophil counts

The mean heterophil count in the *E. coli*-infected and control layers is presented in Figure 4.9 and Appendix 3. The mean heterophil count showed a rapid and progressive increase in the infected group from day 2 ($2.41 \pm 0.77 \times 10^9$L) to a peak level ($5.08 \pm 0.76 \times 10^9$L) on day 6 pi that was significantly ($p < 0.05$) higher than the corresponding value in the control group. Following this, the value progressively decreased in the infected but remained significantly ($p < 0.05$) higher than in the control up to the termination of this experiment.

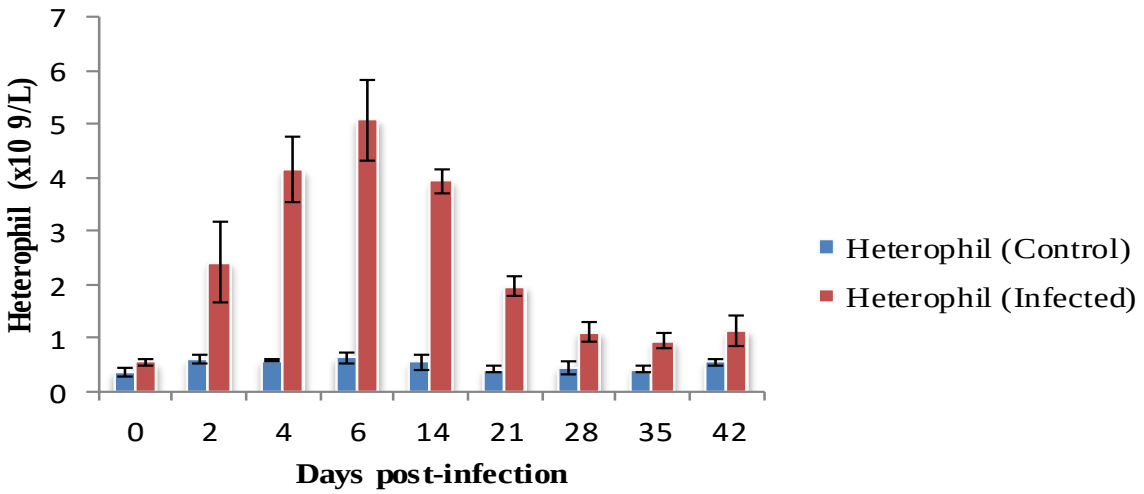

Figure 4.9: Mean (± SEM) heterophil count in the *E. coli*-infected and control groups of layers

4. 5.8 Lymphocyte counts

The mean lymphocyte count in the *E. coli*-infected and control layers is presented in Figure 4.10 and Appendix 3. The mean lymphocyte count progressively increased starting from day 4 pi (16.47±1.78x 10^9/L) to the highest value (21.59 ± 0.92 x 10^9/L) on day 14 pi that was significantly ($p< 0.05$) higher than the control group. The mean lymphocyte count remained significantly higher than that in the control group, up to day 42 pi when the experiment was terminated.

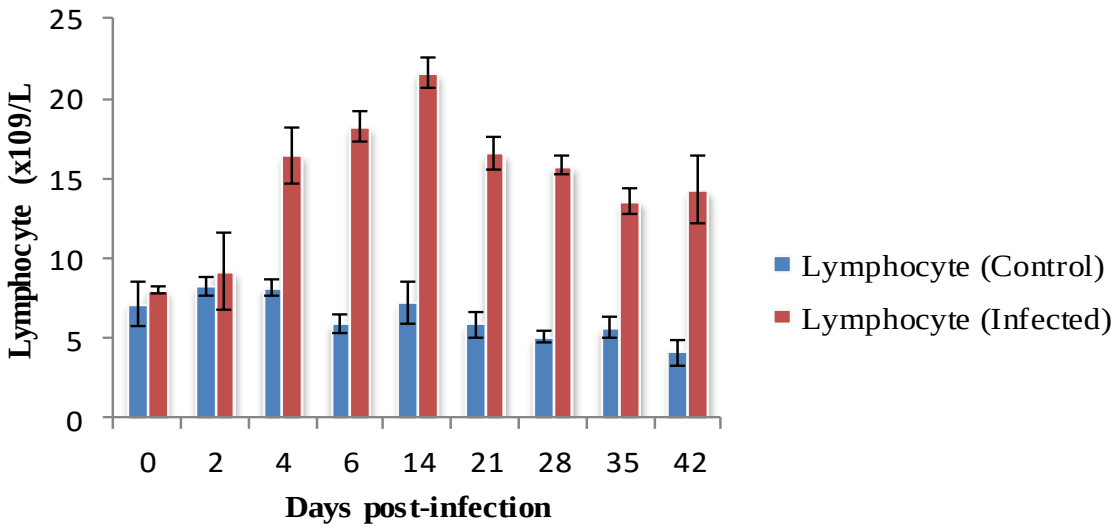

Figure 4.10: Mean (± SEM) lymphocyte count in the *E. coli*-infected and control groups of layers

4. 6 Effect of *E. coli* Infection on the Endocrine and Biochemical Parameters of Layers.

4. 6.1 Plasma calcium concentration

The mean plasma calcium concentrations in the *E. coli*-infected and control layers are presented in Figure 4.11 and Appendix 4. Plasma level of calcium in the infected group remained relatively unchanged and comparable with that of the control group up to day 4 pi. A drop in the plasma calcium level was then observed beginning from day 6 (2.24 ± 0.05 mmol/L) to its lowest level (1.61 ± 0.11 mmol/L) on day 14 pi, which was significantly ($p<$ 0.05) different from that of the control group. Plasma calcium concentration in the *E. coli*-infected group then showed slight increase to a level that was still lower than that of the control group and remained so up to termination of the experiment. The plasma calcium concentration in the control group was maintained at fairly the same level with only some non-significant fluctuations.

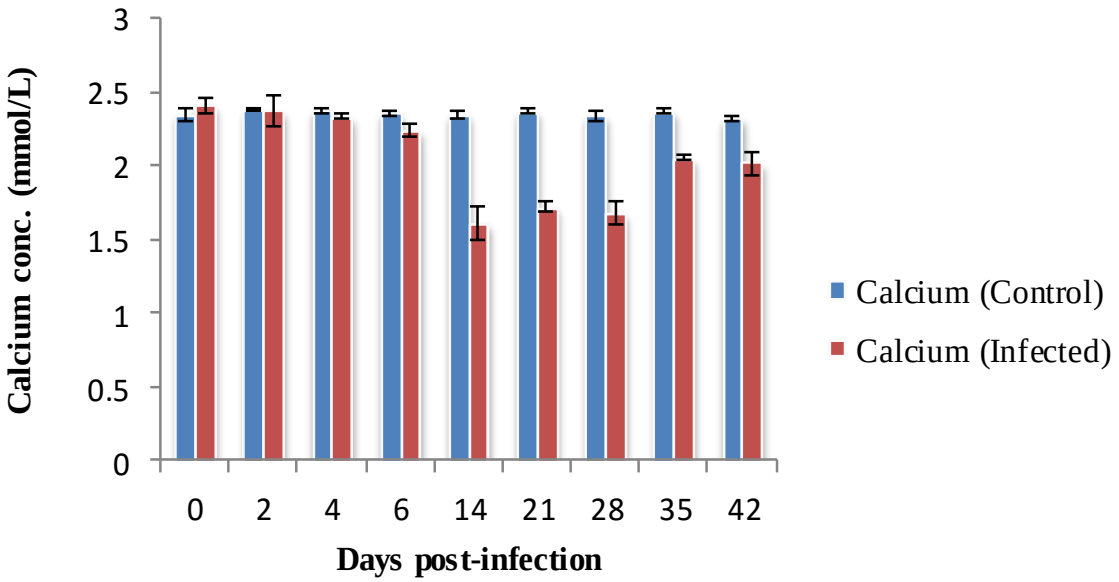

Figure 4.11: Mean (± SEM) Calcium Concentration in *E. coli*-infected and control groups of layers

4. 6.2 Plasma inorganic phosphate concentration

The mean plasma phosphate concentrations in the *E. coli*-infected and control layers are presented in Figure 4.12 and Appendix 4. The mean plasma phosphate concentrations in the infected and control groups were comparable up to day 6 pi, following which a sharp increase was observed in the *E. coli*-infected group, on day 14 pi, to a significantly ($p< 0.05$) higher level (1.64±0.12) than in the control group. The mean plasma phosphate concentration in the infected group was, thereafter, maintained at higher levels compared to those of the control up to the end of the experiment.

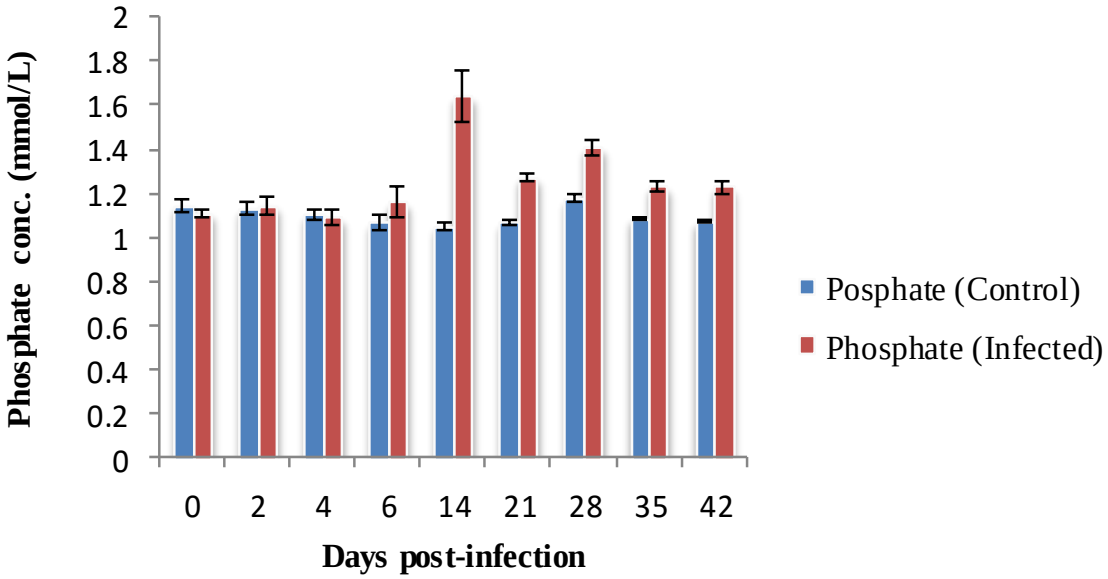

Figure 4.12: Mean (± SEM) plasma phosphate concentration in *E. coli*-infected and control groups of layers

4. 6 .3 Plasma parathormone profile

The mean plasma parathormone profile in the *E. coli*-infected and control layers are presented in Figure 4.13 and Appendix 4. The mean plasma parathormone profiles in the infected and control groups were fairly at the same levels up to day 6 pi. A significant ($p<$ 0.05) increase in the mean profile of this hormone was observed in the infected group, from day 6 value of 24.10 ± 0.56 pg/ml to 27.40 ± 0.79 pg/ml on day 14 pi, and peaked (31.04±0.80 pg/ml) on day 21 pi before it gradually dropped to a level comparable with that of the control group on day 42 pi. The mean plasma parathormone profile in the control group remained relatively unchanged throughout the experiment.

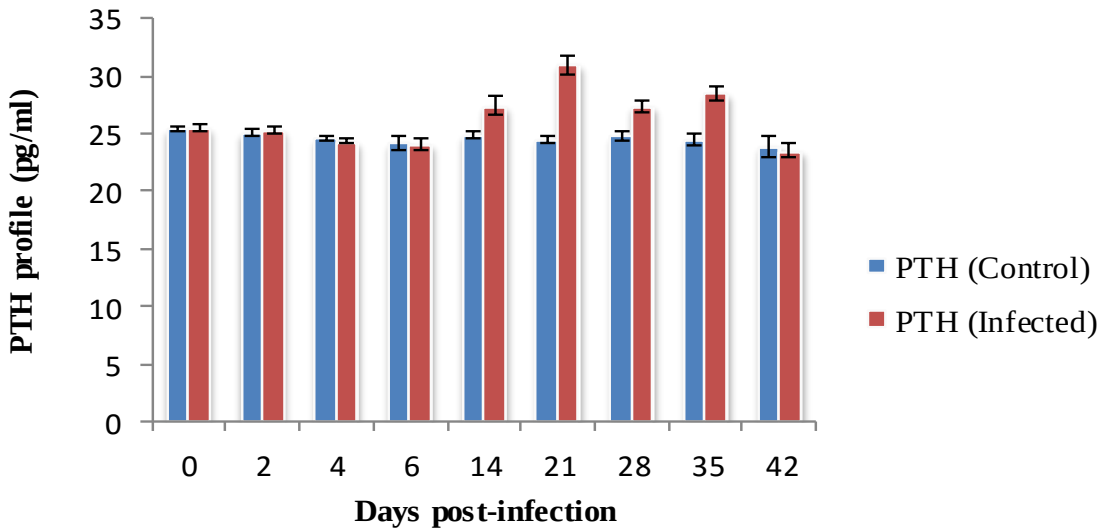

Figure 4.13: Mean (± SEM) Parathormone (PTH) profile in *E. coli*-infected and control groups of layer

4. 6. 4 Plasma oestrogen profile

The mean plasma oestrogen profile in the *E. coli*-infected and control layers are presented in Figure 4.14 and Appendix 4. The mean plasma oestrogen profile in the infected group was comparable to that of the control birds up to day 14 pi following which a progressive decrease from 347±2.55 to a lowest value (332.6±5.41pg/ml) on day 28 pi was observed that differed significantly ($p< 0.05$) from the corresponding value in the control group. The mean plasma oestrogen profile, thereafter, was maintained at significantly lower levels than the corresponding control values up to end of the experiment.

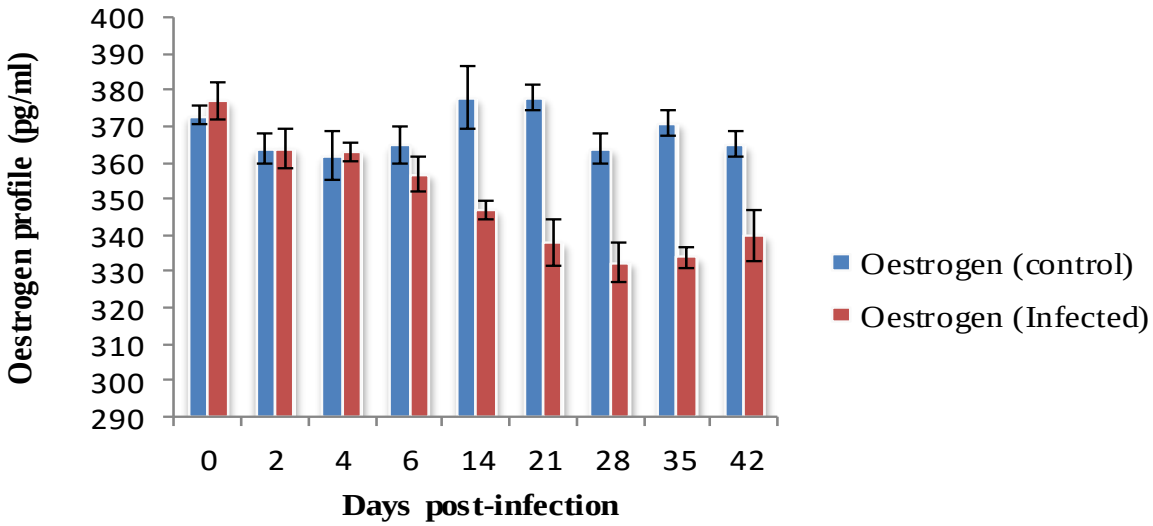

Figure 4.14: Mean (± SEM) Oestrogen profile of *E. coli*-infected and control groups of layers

4. 6. 5 Plasma progesterone profile

The mean plasma progesterone profile in the *E. coli*-infected and control layers are presented in Figure 4.15 and Appendix 4. The mean progesterone profile in the infected group progressively decreased starting from day 4 pi (117.1 ± 2.98 pg/ml) to a lowest value (94.8 ± 1.98 pg/ml) on day 28 pi that differed significantly ($p<0.05$) from that in the control group. The low levels of this hormone were maintained in the infected group up to day 42 pi when the experiment was terminated.

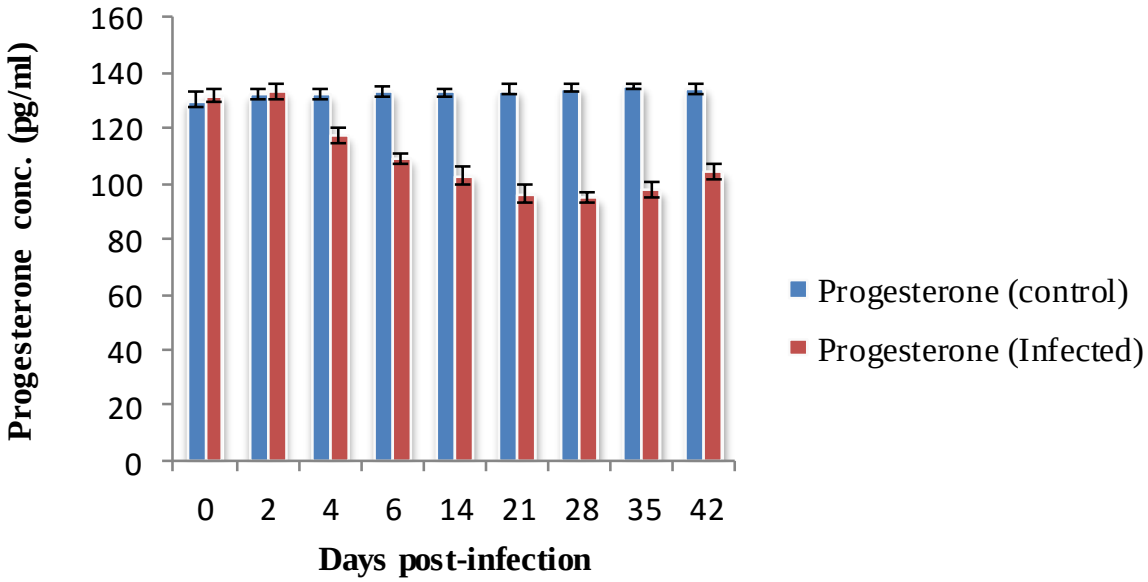

Figure 4.15: Mean (± SEM) Progesterone profile in *E. coli*-infected and control groups of layers

4.6.6 Plasma aspartate aminotransferase (AST) activity

The mean AST activity in the *E. coli*-infected and control layers are presented in Figure 4.16 and Appendix 4. The mean plasma AST activity in the infected group progressively increased from day 6 pi (59.2±1.20 IU/L) to a significant ($p< 0.05$) value (66.4 ± 0.93 IU/L) on day 28 pi when compared with those in the control group. The mean plasma values in the infected group remained higher than that in the control up to day 42 when this experiment was terminated.

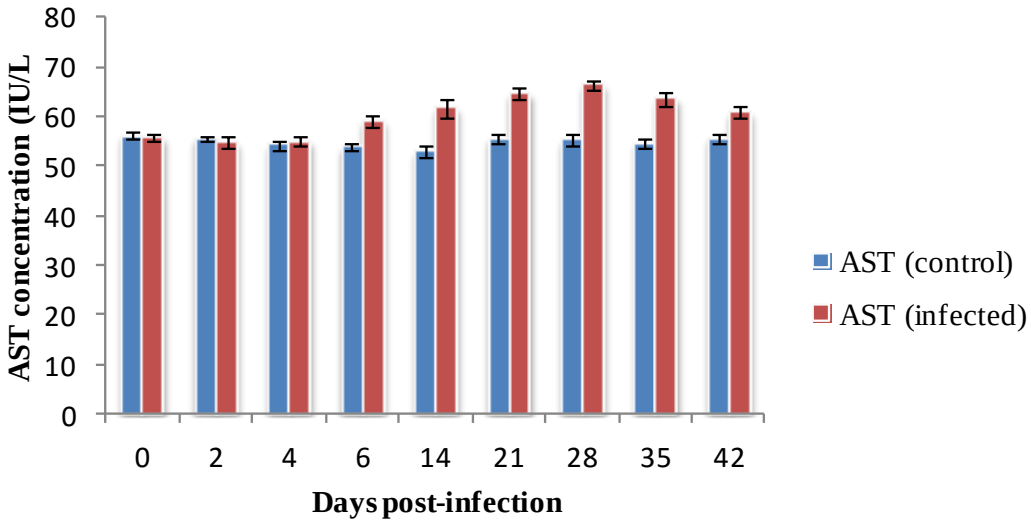

Figure 4.16: Mean (± SEM) aspartate aminotransferase activity in *E. coli*-infected and control groups of layers

4.6.7 Plasma alanine aminotransferase (ALT) activity.

The mean ALT activity in the *E. coli*-infected and control layers are presented in Figure 4.17 and Appendix 4. The mean plasma ALT activity progressively increased in the infected group beginning from day 6 pi (70.2±1.2 IU/L) to a higher value (74.4±2.11 IU/L) on day 21 pi that differed significantly ($p<0.05$) from that of the control group . The mean ALT activity in the infected group remained relatively higher until termination of the experiment.

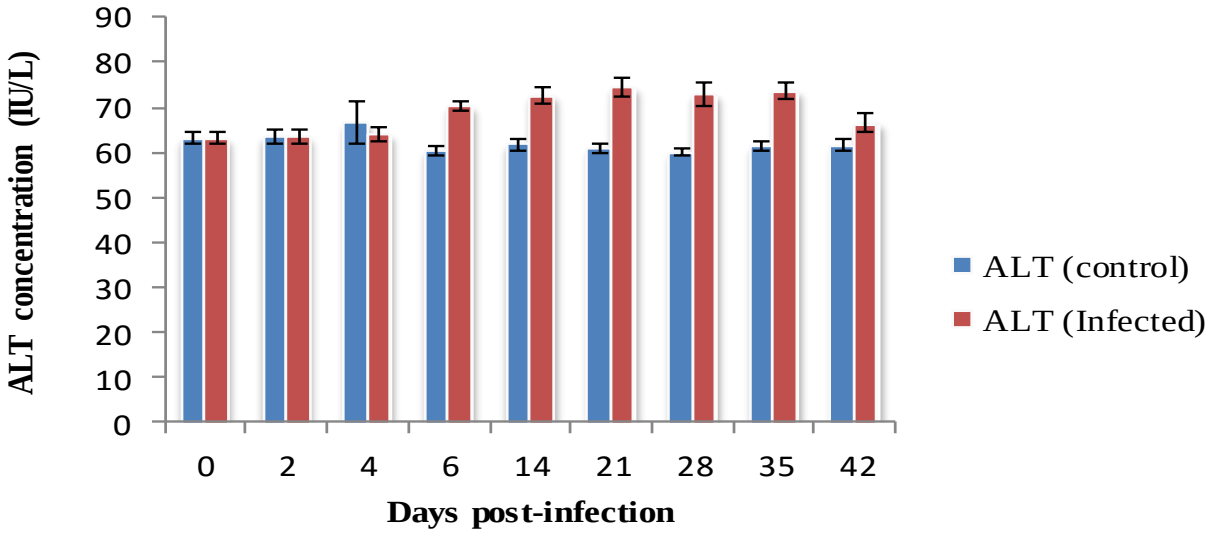

Figure 4.17: Mean (± SEM) alanine aminotransferase activity of *E. coli*-infected and control groups in layers

4.6.8 Plasma total protein Concentration

The mean plasma total protein concentration in the infected group progressively decreased starting from day 4 pi (6.04 ± 0.32 mg/dl) to a lowest value (5.12 ± 0.28 mg/dl) on day 35 pi that differed significantly ($p<0.05$) from that in the control group. The low levels of plasma total protein concentration were maintained in the infected group up to day 42 pi when the experiment was terminated.

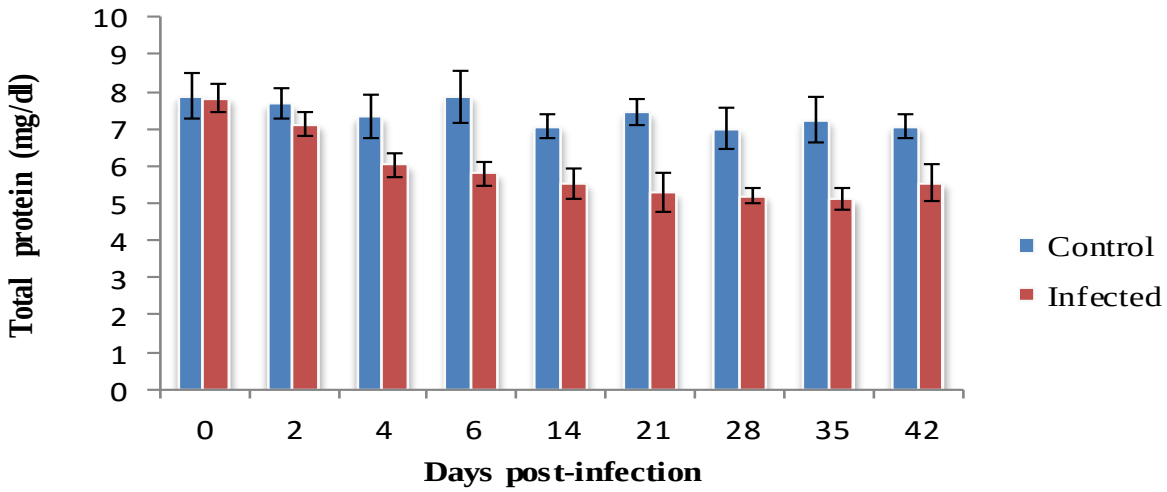

Figure 4.18: Mean (± SEM) total protein concentrations of *E. coli*-infected and control groups in layers

4.7 Gross findings

The gross lesions observed in the *E. coli*-infected layers were congested lungs (Plate III), enlarged liver (Plate IV), hyperaemic intestine (Plate IV), enlarged kidney (Plate V) and congested ovarian follicles (arrow head), yellowish fibrinous material (short arrow) congested blood vessels of the oviduct (Plate V).

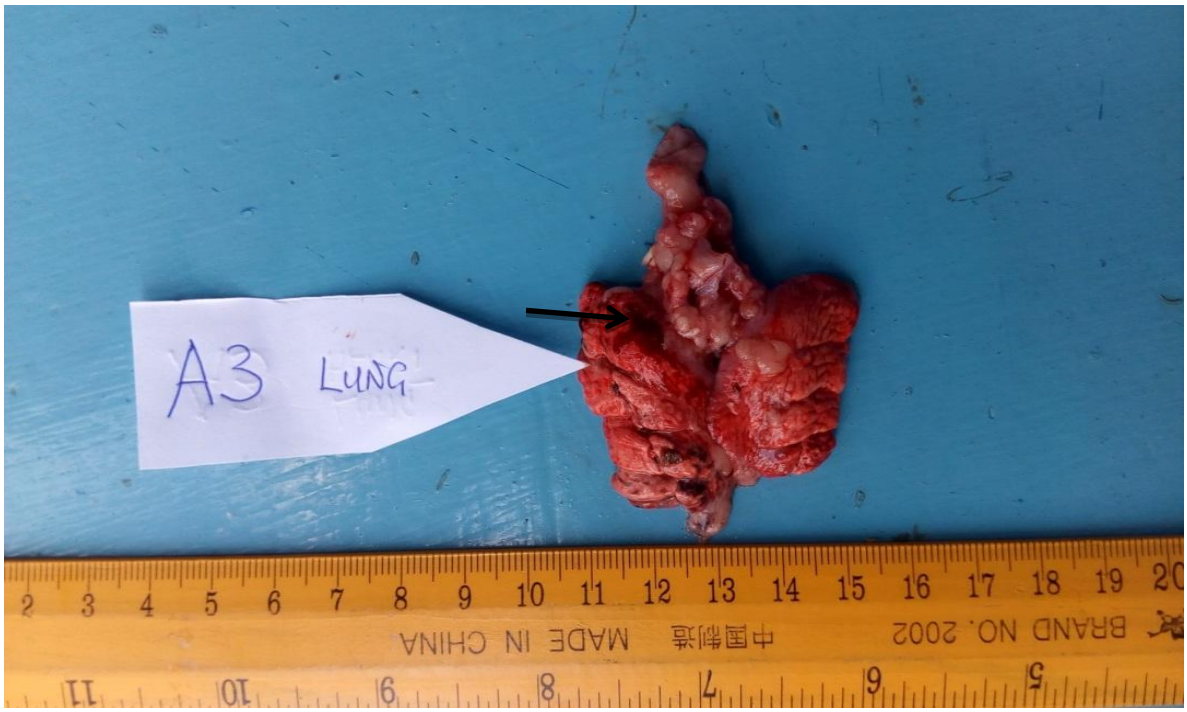

Plate III: Congested Lungs (arrow) from a layer in the *E. coli*-infected group on day 10 pi.

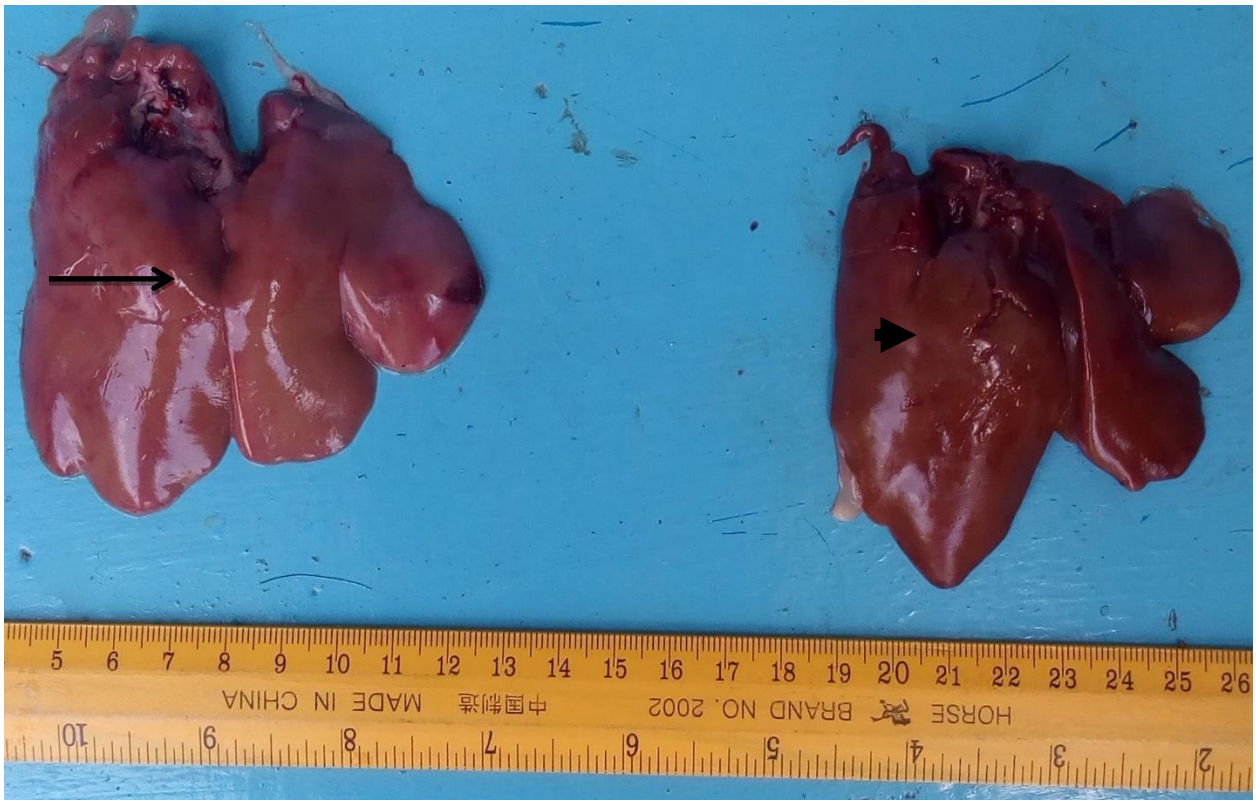

Plate IV: Enlarged liver (arrow) from *E. coli*-infected layer and liver from the control group (arrow head) on day 10 pi.

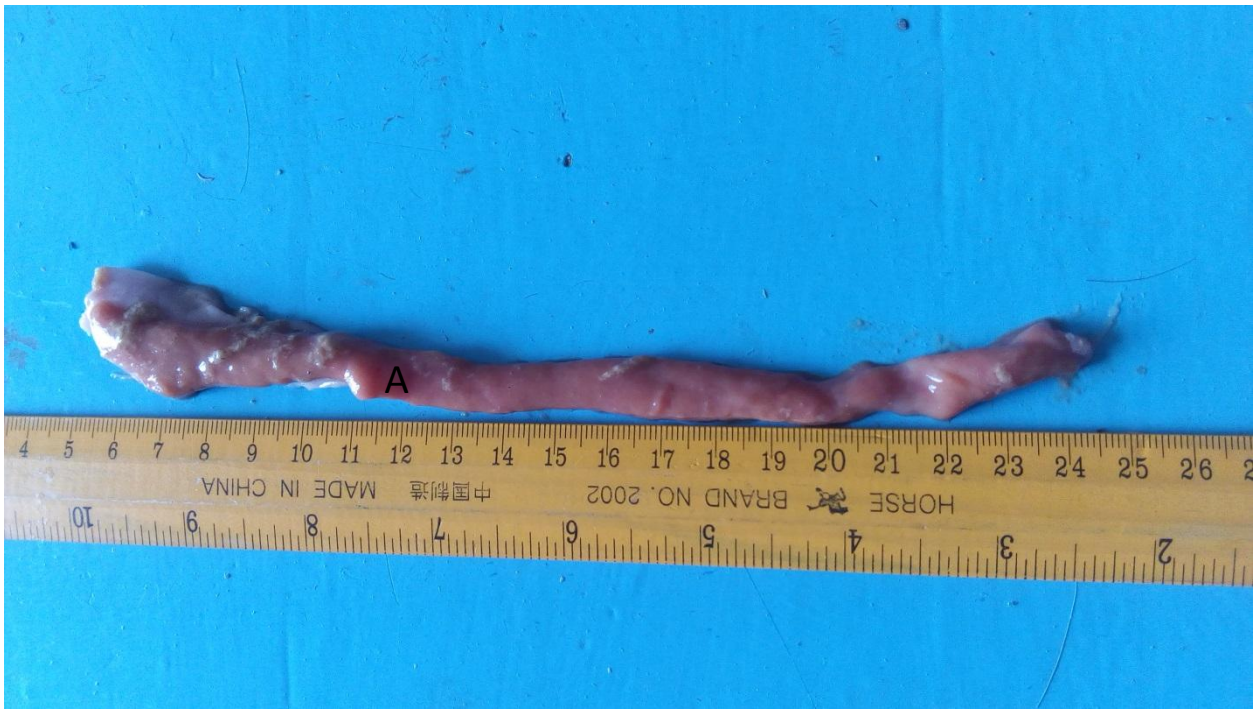

Plate V: Intestine showing hyperaemia (A) and thickened mucosal membrane from *E. coli*-infected layer on day 10 pi.

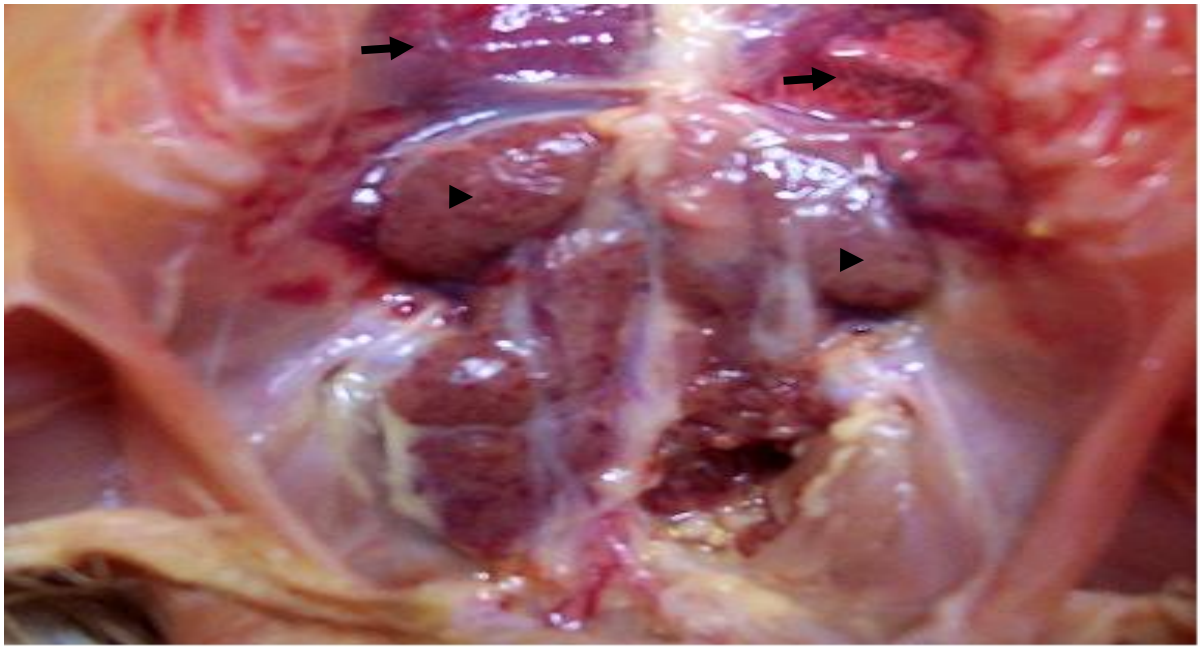

Plate VI: Congested lungs (short arrow) and enlarged kidney (arrow head), from *E. coli*-infected layer on day 35 pi.

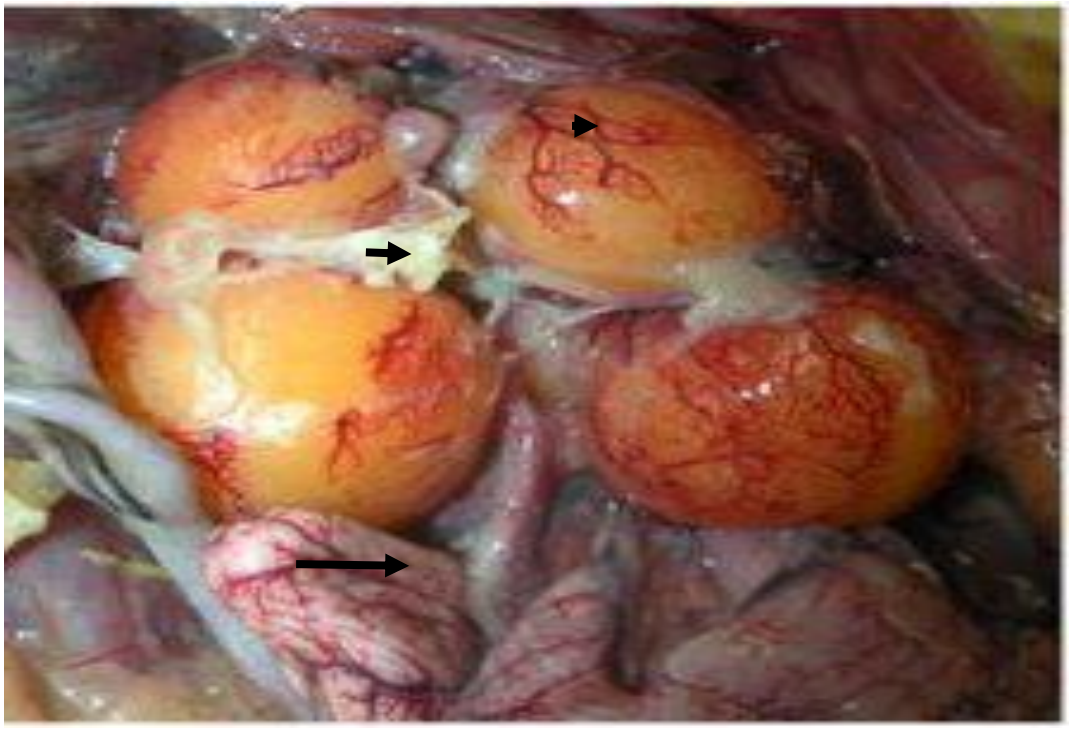

Plate VII: Congested ovarian follicles (arrow head), yellowish fibrinous material (short arrow) congested blood vessels of the oviduct (long arrow) in the *E. coli*-infected layer on day 35 pi.

4.8 Histopathology of tissues harvested from *E. coli*-infected group of layers.

The ovarian follicle showed congestion of ovarian blood vessels (Plate VIII). Heart showed necrosis of cardiac myocytes, congested blood vessels, mononuclear cellular infiltration (Plate IX). The kidney showed diffused necrosis of renal tubular epithelium, aggregation of mononuclear cells and congested blood vessels (Plate X). Liver showed diffused necrosis of hepatocytes, perivascular aggregation of mononuclear cells and congestion of the central vein (Plate XI).

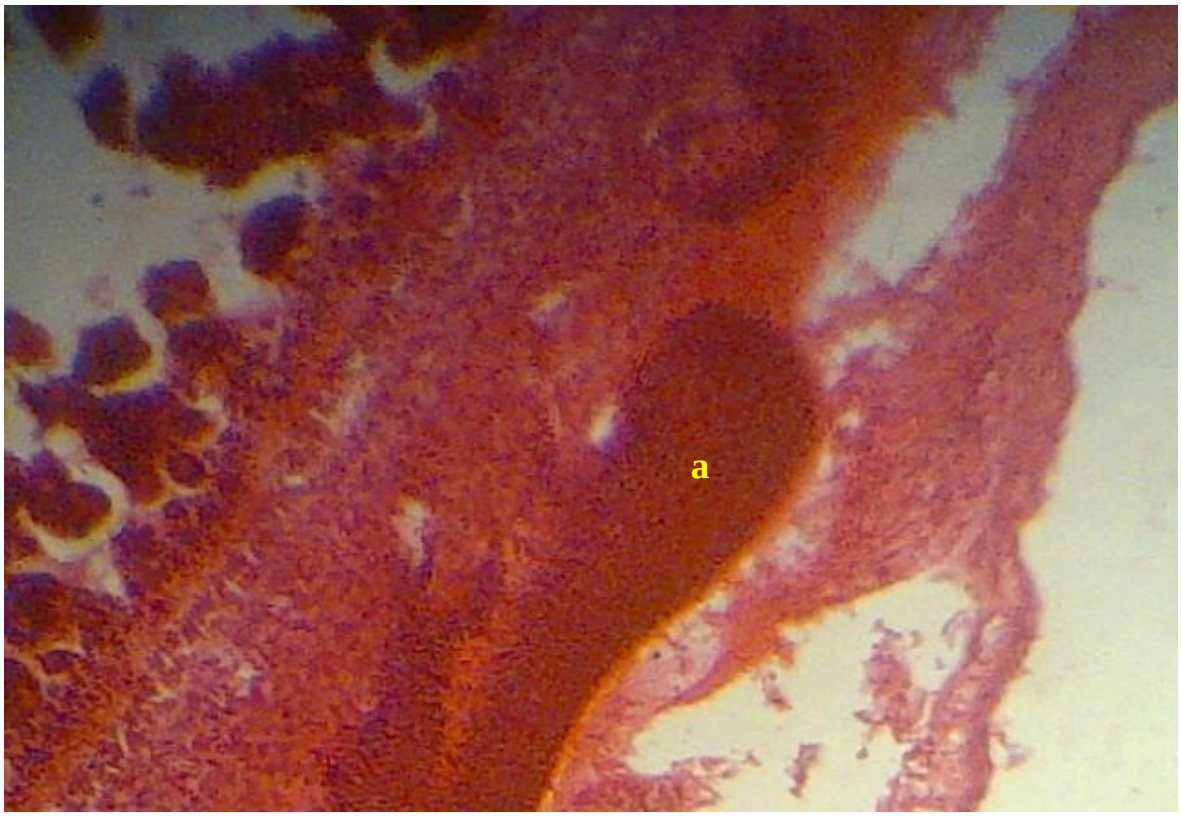

Plate VIII: Photomicrograph of ovary section from *E. coli*-infected chicken showing (a) congestion of ovarian blood vessel on day 35 pi. H&E X 250

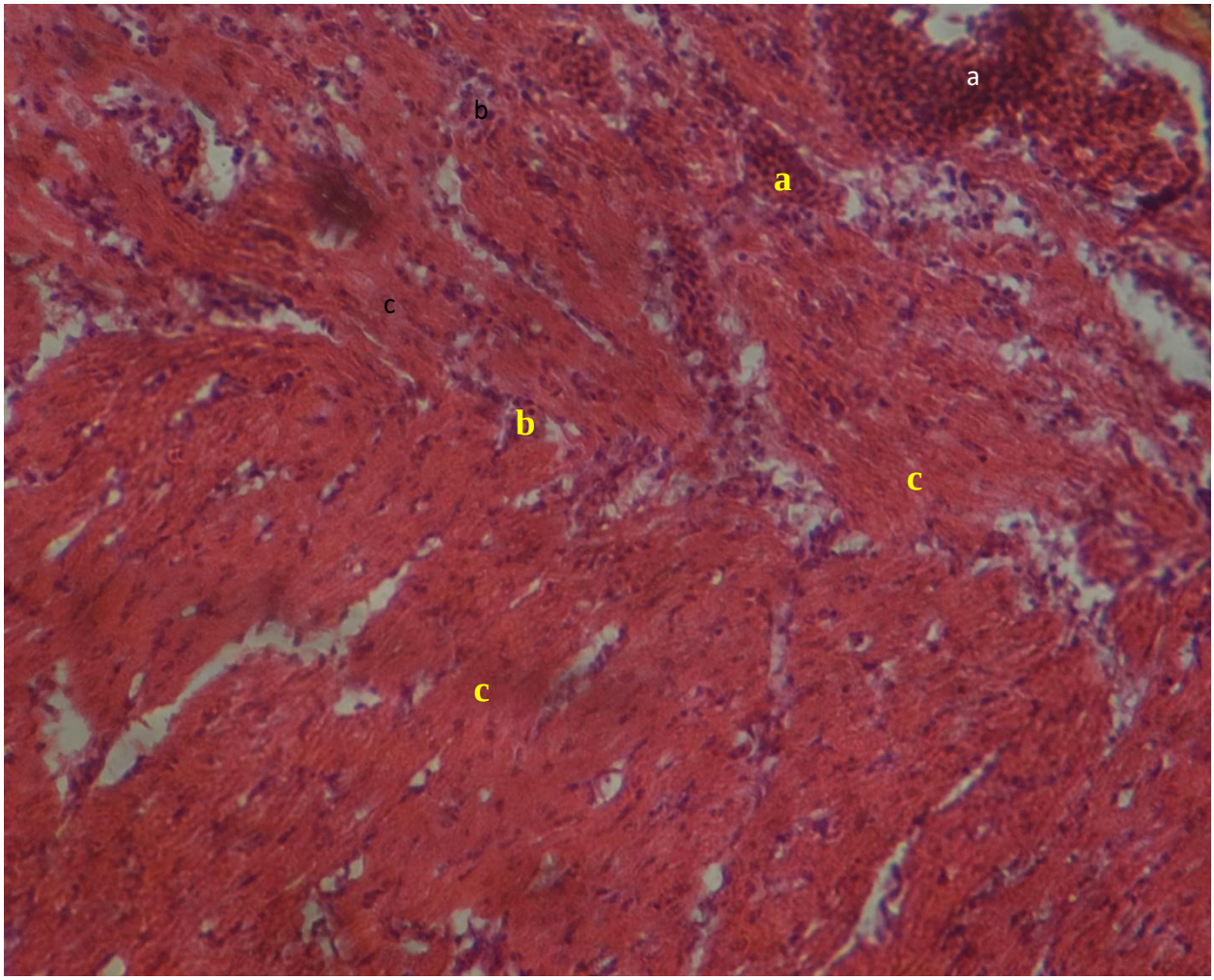

Plate IX: Photomicrograph of section of heart from *E. coli*-infected chicken showing congestion (a), mononuclear cellular infiltration (b), necrosis of the cardiac myocytes (c) on day 35 pi. H&E X 250.

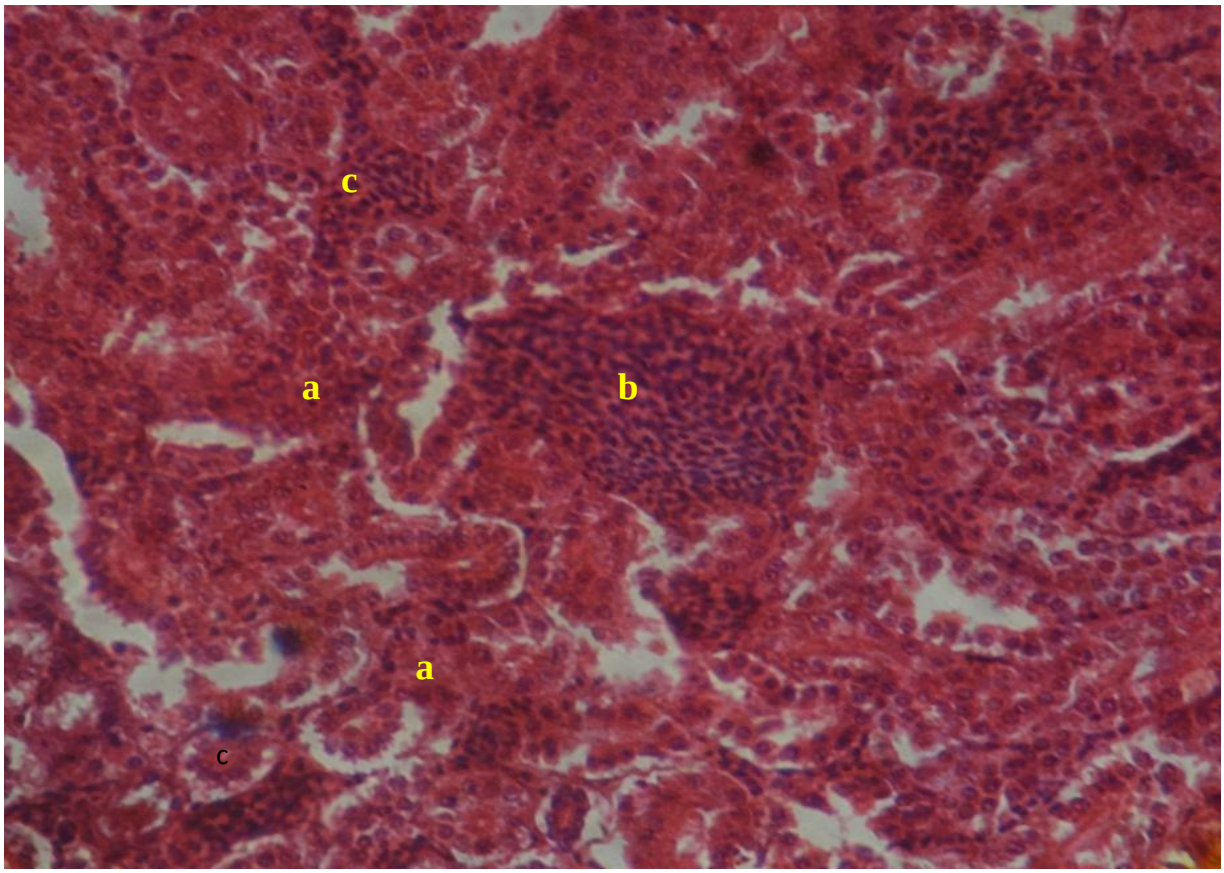

Plate X: Photomicrograph of section of kidney from *E. coli*-infected chicken showing diffused necrosis of renal tubular epithelium (a), aggregation of mononuclear cells (b), congested blood vessels (c) on day 35 pi. (H&Ex250)

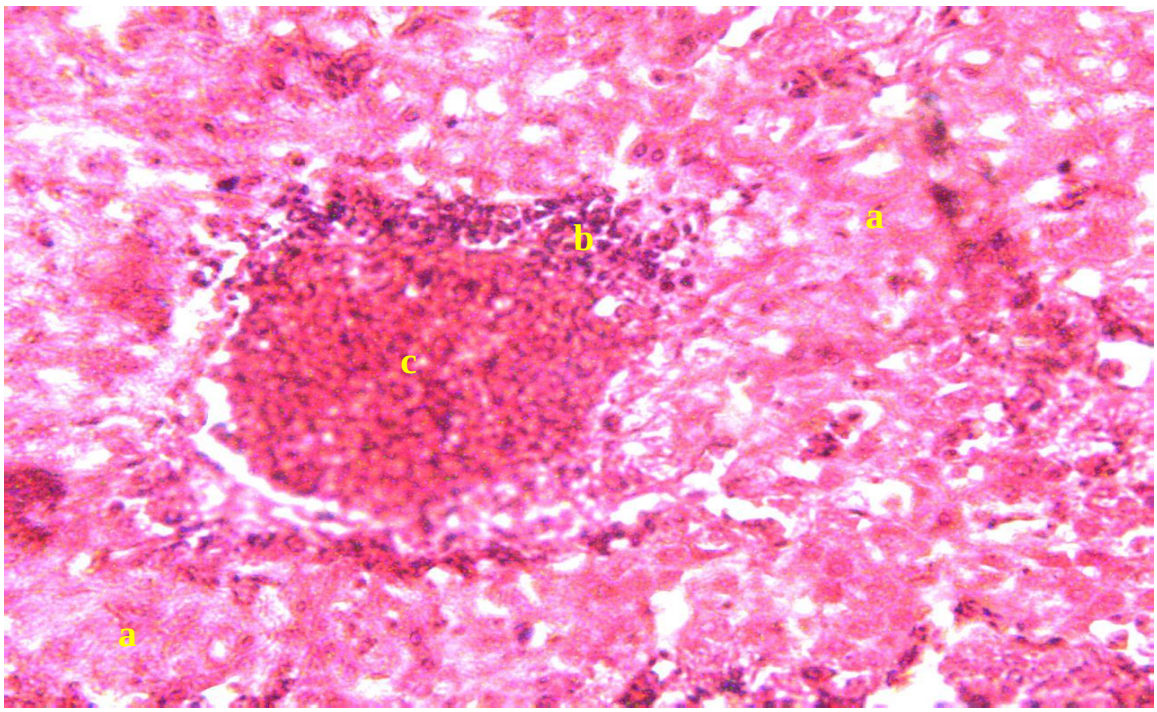

Plate XI: Photomicrograph of liver section from *E. coli*-infected chicken showing diffuse necrosis of hepatocytes (a), perivascular aggregation of mononuclear cells (b), congestion of central vein (c) on day 35 pi. H&E x 400

CHAPTER 5

5.0 DISCUSSION

The clinical signs observed in the *E. coli*-infected layers in this study, namely, reduced feed and water consumption, decreased egg production, watery yellowish faeces, weakness and loss of weight by this organism were the classical findings in colibacillosis (Barnes *et al.*, 2008). The gross lesions observed in the *E. coli*-infected layers such as the congested lungs, congested serosal blood vessels of the oviduct, congested ovarian follicles with yellowish fibrinous materials and hyeraemic intestine were also part of the reported postmortem findings in birds with colibacillosis (Tonu *et al.*, 2011; Srinivasan *et al.*, 2013).

The reduced feed intake and loss of body weight observed in this study may be part of the acute inflammatory response immediately after *E. coli* contacts host tissues and partly due to the effects of acute phase of endotoxaemia (Mireles *et al*., 2005). This is a non-specific response mediated by acute phase proteins produced in the liver; cytokines such as IL-1, IL-6 and tumour necrosis factor were reported to increase rapidly following exposure to endotoxins (Nakamura *et al*., 1998, Chamanza *et al.*, 1999; Xie *et al.*, 2002). The loss of body weight observed in the infected group of layers may be as a result of combined effects of reduced feed intake and the diarrhoea due to enteritis, which could have impaired digestion as well as intestinal absorption of the nutrients needed for normal body functions and reproduction in layers (Franco-Jimenez and Beck, 2007; Abdu, 2014).

The decrease in egg production may be due to decreased progesterone levels which was evident in this study. This is because it has been reported that progesterone is responsible for the preovulatory Luteinizing hormone (LH) surges and induction of ovulation (Sturkie, 1970; Johnson, 2014). The decrease in egg production may also be due to the hampered uptake of gut calcium (Franco-Jimenez and Beck, 2007) or the decreased production of cholecalciferol-

1-hydroxylase, which has a momentous role in calcium homeostasis (Elaroussi *et al*., 1994). Although the activity of cholecalciferol-1-hydroxylase was not investigated in this study, decreased production of this enzyme could negatively affect calcium metabolism with consequent effect on egg production.

The decrease in PCV, RBC count and haemoglobin concentration was in agreement with findings in the reports of Christie and Halliday (1979) and Saini (2004). The former of these authors observed fall in packed cell volume, haemoglobin level and red cell count, indeed, 24 hours post-inoculation of birds with the organism. Many factors could have contributed to the reduction in the haematological parameters. It has been suggested that septicemia produced by *E. coli* (Christie and Halliday, 1979) could produce haemodilution which is reflected as reduction in the parameters. Also, these observed changes may also be due to reduction in supply of nutritional factors necessary for sustenance of normal erythropoiesis (Esievo, 2017; Ogungbemi *et al.,* 2017) as consequence of anorexia and diarrhoea (Feldman *et al*., 2000) observed in the *E. coli*-infected group.

The significantly higher mean corpuscular volume recorded in the *E. coli*-infected layers in this study on day 14 pi signified a possible macrocytic anaemia. Since the diarrhoea observed in the *E. coli*-infected birds in this study was not haemorrhagic, it is only reasonable to think that a deficiency of some vitamins and minerals, such as B_{12}, folic acid and cobalt that are essential for normal erythropoiesis maybe responsible for the macrocytosis (Ogungbemi *et al*., 2017), which could have occurred due to both reduced feed intake and possible impairment of intestinal absorption as a result of diarrhoea. The finding that MCHC values did not show any significant difference between *E. coli*-infected and control group is in variance with the report of Haq *et al.* (2015), who reported a decrease in MCHC in *E. coli* infection in pigeon. The absence of significant difference in the MCHC values of the *E. coli*-

infected and control layers further lends credence to the earlier suggestion that reduction in erythrocyte parameters observed in this study was due to deficiency of the nutritional factors as discussed earlier. So the anaemia observed in this study was macrocytic normochromic anaemia.

The finding that the leucogram of the *E. coli*-infected layers showed significant increase in mean WBC, heterophil and lymphocyte counts in the early phase of the infection is in line with reports on WBC count changes in bacterial infections (Honda *et al.,* 2016). Some of the most common causes of increase in WBC count are localized or generalized infections, tissue necrosis, acute haemorrhages and haemolysis (Morgulis, 2002; Benjamin, 2013; Esievo, 2017). As such, the significant increase in the WBC count in the *E. coli*-infected layers, observed on day 2 up to 42 pi in the present study could be attributable to the bacterial infection. The increase in the WBC count in the *E. coli*-infected layers as observed in this study was also due to the significant increase in heterophil and lymphocyte counts.

The significant increase in the mean heterophil count in the *E. coli*-infected layers, observed right from day 2 concurs with the fact that these phagocytes are the first cells to migrate to the site of infection where they engage in phagocytosis and killing of pathogens by producing toxic reactive oxygen species and releasing bactericidal substances and proteolytic enzymes in the process of oxidative burst and degranulation (He *et al*., 2008; Genovese *et al*., 2013). Heterophils are known to increase in circulation in both local and generalized infections and septicemias, of which *E. coli* is one of the most common causes (Maxwell and Robertson, 1998; Morgulis, 2002). Migration of heterophils into sites of infection or inflammation was thought to place the demand for increased bone marrow haematopoietic tissue to increase production of heterophil precursor cells (Campbell and Coles, 1986; Irizarry-Rovira, 2004; Juul-Madsen *et al*., 2008).

The significant increase in the mean lymphocyte count in the *E. coli*-infected layers, observed on days 4 to 42 pi suggests that infection with *E. coli* stimulates lymphocytopoiesis. Mitchell and Johns (2008) reported that an increased presence of lymphocytes in peripheral circulation was commonly seen in birds with infectious diseases. Thus, infection with *E. coli* may be associated with significant antibody production and may be a mark of recovering of the birds from infectious disease (Campbell, 2004a).

The significant decrease in the mean plasma concentration of calcium observed in the *E. coli*-infected layers, especially on days 14, 21 and 28 and with the level remaining significantly lower than in the uninfected control up to termination of the experiment is a pointer to the fact that colibacillosis could be associated with some derangement in the metabolism of calcium. It was probable that the observed enteritis in the *E. coli*-infected layers in this study had interfered with either feed digestion or subsequent malabsorption of nutrients as earlier reported (Yegani and Korver, 2008; Hogenauer and Hammer, 2016), including the calcium, thus contributing to the lowered levels in the infected group. It was also probable that the observed decline in mean plasma oestrogen activity had contributed to the lowering of plasma calcium concentration observed in this group of layers. This is because oestrogen plays a role in hepatic synthesis of vitellogenins, which are lipoproteins that bind calcium; a rise in their concentration is associated with a rise in plasma calcium concentration and vice versa (Bentley, 1998; Dacke, 2000). Thus, the observed liver lesions such as generalised necrosis of hepatocytes evident in this study could affect the liver synthesis of these proteins. Also, the observed kidney lesions such as renal tubular necrosis and congested blood vessels could have affected both calcium and phosphorus metabolism. This is because the kidney is known to be site of conversion from inactive to active form of vitamin D, which enhances

renal and intestinal absorption of calcium (Beer *et al*., 1985; Campell and Coles, 1986; Mark and Robert, 1993; Hamdi, 2016).

The finding that the mean plasma phosphates concentration was significantly increased in the *E. coli*-infected layers from day 14 up to 42 pi in the face of lowered mean plasma calcium concentration was pathologic, since the body physiology dictates that plasma calcium concentration is altered alongside that of phosphates (Kirsten*et al.,* 2008; Pelicia *et al.,* 2009, Blaine *et al.,* 2015). This change suggests some impairment of renal excretion of phosphates, since in normal health, phosphorus is excreted in urine as part of effort to maintain calcium-phosphorus ratio (Evenepoel and Wolf, 2013).

The finding that the response to increase in the mean parathormone profile, which started on day 14 pi, was only noticeable on day 35 pi suggests impairment at the hormone's target cells. The observed renal tubular lesions might have been responsible for delayed response to the parathormone profile. This is because it has been reported that in renal failure, phosphate loading or hyperphosphataemia decreases the calcaemic response to parathormone activity (Somerville and Kaye, 1979; 1982; Rodriguez *et al.,* 1991). The significant increase in the mean plasma parathormone profile is believed to be physiological response to the significant decrease in plasma calcium (Brommage *et al.,* 1999; Murray *et al.,* 2005).

The significant decrease in the mean plasma oestrogen profile from day 14 up to 42 pi, observed in the *E. coli*-infected layers, suggests impairment of its secretion from the ovary. Indeed, the observed progressive decrease in mean plasma progesterone profile from days 4 to 42 pi, which coincided with the period of decline in oestrogen profile, was supporting evidence that the infection with *E. coli* resulted in impairment of some ovarian functions, probably due to ovarian lesions. The observed congestion of the ovarian follicles in this

study strongly supports this hypothesis. *E. coli* infection-induced changes in ovarian follicles were similarly reported by Oh *et al.* (2011) and Srinivasan *et al.* (2013). The significant decrease in the mean plasma progesterone profile in the *E. coli*-infected layers may also be due to lower feed intake which has an important correlation with circulating progesterone contents (Lei *et al.*, 2014) and disappearance of pre-ovulatory follicles after a few days of feed withdrawal (Gilbert and Wells, 1984).

The grossly observed congestion of the serosal blood vessels of the oviduct was previously reported following *E. coli* infection of layer birds (Bisgaard and Dam, 1980; Srinivasan *et al*., 2013). It has been reported that lesions associated with ascending *E. coli* infection from the cloaca consequent of salphingitis (Bisgaard and Dam 1981; Landman and Cornelissen, 2006; Ozaki and Murase, 2009) could be responsible for egg peritonitis frequently encountered in layer birds (Dinev, 2018). Peritonitis in this case, and as reported, follows a reverse movement from the oviduct into the abdomen of albumen and *E. coli* bacteria (Gebremichael and Darge, 2017).

The increase in the mean plasma AST activity is indicative of cellular injury to hepatocytes and cardiac myocytes, while the elevated plasma ALT activity was most probably due to presence of the hepatic lesions observed in the *E. coli*-infected layers in the present study. Similar increases in plasma ALT and AST activities in *E. coli*-infected chickens were earlier reported by Rath *et al.* (2017). Hepatic damage could lead to impaired synthesis of vitellogenins, which could have contributed to the lowering of the plasma calcium concentration in the *E. coli*-infected group. Increases in plasma activity of ALT and AST were also reported by Bokori and karasi (1969) and Omaima (1987). The observed significant decrease in the mean total plasma protein concentration in the *E. coli*-infected group in this study may partly be attributed to impaired synthesis of these proteins due to the *E. coli*

infection-induced hepatic damage (Oladele *et al.*, 2005). Malnutrition, resulting from anorexia (Petersen *et al.,* 2001) and impaired digestion and malabsorption of nutrients from the intestinal tract could have played a part in the observed decrease in total plasma protein.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusions

It could be concluded from this study that the experimental infection with *Escherichia coli* in layers caused:

i. the full blown disease, colibacillosis, with its characteristic clinical manifestations (reduced feed and water consumption, decreased egg production, watery yellowish faeces, weakness) evident by day 3 post-infection;

ii. a progressive decrease in mean plasma oestrogen activities from day 14 post-infection value of 347 ± 2.55 pg/ml to a significantly ($p< 0.05$) low level (332.6 ± 5.41 pg/ml) on day 28 post-infection, when compared to the corresponding value (364.3 ± 4.30 pg/ml) in the control group.

iii. a progressive decrease in mean plasma activity of progesterone from day 4 value of 117.1 ± 2.98 pg/ml to a significantly ($p< 0.05$) low level (94.8 ± 1.98 pg/ml) on day 28 post-infection, when compared to the corresponding value (134.8 ± 1.36 pg/ml) in the control group;

iv. an increase in mean plasma parathormone activity from day 14 to reach peak level on day 21 (31.04 ± 0.80 pg/ml) that differed significantly ($p < 0.05$) relative to that (24.5 ± 0.32 pg/ ml) in the control;

v. decrease in mean plasma calcium concentration from day 6 value of 2.38 ± 0.03 mmol/L to a significantly ($P < 0.05$) low level (1.61 ± 0.11 mmol/L) on day 14 post-infection, when compared to the corresponding value in the control;

vi. an increase in mean plasma aspartate aminotransferase and alanine aminotransferase activities that differed significantly ($P < 0.05$) relative to that of the control;

vii. significant decrease in packed cell volume, relative to the control, with erythrocyte morphology being macrocytic normochromic;

viii. a rapid increase in total white blood cell count from day 2 value of 14.81 ± 3.28 x 10^9/L to reach a peak level (29.16 ± 0.81 x 10^9/L) on day 14 pi that differed significantly ($P < 0.05$) relative to that of the control.

6.2 Recommendations

- More studies should be carried out to establish the patho-physiological mechanisms of *E. coli* infection-induced reproductive endocrine disorders.
- More studies should be carried out to determine the effect of *E. coli* infection on hepatic synthesis and plasma levels of vitellogenins, which bind calcium and plays vital role during egg shell formation in chickens.
- Since *E. coli* infection could lead to lowered egg production, biosecurity measures should be well instituted in poultry farms to minimize the associated losses due to colibacillosis.

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APPENDIX 1

Mean body weight in *E. coli*-infected and control layers

Day	Control	Infected
0	1642 ± 4.64	1649 ± 10.30
2	1655 ± 2.55	1635 ± 4.36
4	1800 ± 3.54	1576 ± 44.56
7	1855 ± 5.24	1480 ± 48.89
14	1868 ± 5.15	1490 ± 40.70
21	1879 ± 4.30	1494 ± 43.2
28	1885 ± 4.18	1592 ± 61.02
35	1830 ± 5.84	1792 ± 3.39
42	1892 ± 3.39	1794 ± 2.35

APPENDIX 2

Mean weekly egg production in *E. coli* infected and control layers

Week	Control	Infected
1	70.0 ± 3.09	60.0 ± 3.09
2	77.1 ± 1.84	48.6 ± 3.40
3	78.6 ± 2.61	45.7 ± 2.02
4	80.0 ± 2.18	41.4 ± 2.61
5	82.9 ± 1.84	41.4 ± 1.43
6	84.1 ± 2.02	42.9 ± 1.84

APPENDIX 3

Mean (±SEM) of Haematological Parameters of *E. coli* infected and control groups in commercial layers

		SAMPLING INTERVAL IN DAYS								
		0	**2**	**4**	**6**	**14**	**21**	**28**	**35**	**42**
PCV (%)	Control	25 ±0.71	25 ±0.93[a]	24 ±1.12[b]	26 ±1.46[c]	25 ±0.75[d]	24 ±0.32[e]	25.2 ±1.02	24.6 ±0.81	24 ±0.45[f]
	Infected	23.4 ±0.98	21.2 ±1.41[a]	17.0 ±0.71[b]	19.0 ±1.11[c]	20.8 ±0.91[d]	20.8 ±0.73[e]	24.6 ±1.03	24.0 ±0.45	22.0 ±0.83[f]
RBC (x10$^{12/l}$)	Control	2.12 ±0.03	2.11 ±0.02	2.16 ±0.02[a]	2.42 ±0.21[b]	2.214 ±0.042[c]	2.14 ±0.07[d]	2.21 ±0.18[f]	2.15 ±0.14	2.14 ±0.02
	Infected	2.13 ±0.01	1.92 ±0.04	1.78 ±0.08[a]	1.69 ±0.07[b]	1.63 ±0.07[c]	1.76 ±0.12[d]	2.55 ±0.08[f]	2.20 ±0.18	2.16 ±0.04
Hb (g/dl)	Control	8.33 ±0.24	8.20 ±0.31	7.87 ±0.37[a]	8.73 ±0.49[b]	8.20 ±0.25[c]	8.00± 0.11[d]	8.40 ±0.34	8.20 ±0.27	7.33 ±0.28
	Infected	8.27 ±0.24	7.11 ±0.47	5.67 ±0.24[a]	6.27 ±0.37[b]	6.93 ±0.31[c]	6.93 ±0.24[d]	8.20 ±0.34	8.40 ±0.34	7.30 ±0.28
MCV (fl)	Control	118.27 ±3.87	122.60 ±10.03	109.29 ±5.78	109.69 ±5.95	111.07 ±1.02[a]	115.00 ±4.33	116.43 ±9.06	108.03 ±10.91	111.97 ±2.08
	Infected	116.77 ±3.49	112.62 ±9.97	104.64 ±3.95	111.84 ±6.65	127.54 ±12.26[a]	119.97 ±8.07	105.63 ±5.56	113.03 ±12.02	110.67 ±7.46
MCHC (pg/dl)	Control	33.34 ±0.005	33.33 ±0.006	33.34 ±0.004	31.99 ±1.33	33.33 ±0.006	33.33 ±0.004	32.41 ±0.922	33.30 ±0.03	33.33 ±0.006
	Infected	33.34 ±0.005	33.33 ±0.006	33.34 ±0.004	31.99 ±1.33	33.33 ±0.006	33.33 ±0.004	32.41 ±0.922	33.30 ±0.03	33.33 ±0.006
TWB (X10$^{9/l}$)	Control	9.37 ±0.54	9.16 ±0.45[a]	9.68 ±1.09[b]	7.96 ± 0.74[c]	8.96 ± 1.39[d]	7.08 ± 0.97[e]	7.76 ± 0.42[f]	7.04 ± 0.76[g]	7.80 ± 0.39
	Infected	9.08 ± 0.24	14.81 ± 3.28[a]	19.64 ± 2.13[b]	23.56 ± 1.63[c]	29.16 ± 0.81[d]	21.36 ± 1.14[e]	18.41 ± 0.74[f]	14.04 ± 0.74[g]	15.88 ±2.78
Heterophil (x10$^{9/l}$)	Control	0.35 ±0.09	0.59 ± 0.08[a]	0.58 ± 0.03[b]	0.63 ± 0.10[c]	0.55 ± 0.14[d]	0.41 ± 0.07[e]	0.43 ± 0.11[f]	0.42 ± 0.07[g]	0.55 ± 0.06
	Infected	0.54 ±0.07	2.41 ± 0.77[a]	4.14 ± 0.61[b]	5.08 ± 0.76[c]	3.94 ± 0.22[d]	1.97 ± 0.18[e]	1.11 ± 0.17[f]	0.94 ± 0.16[g]	1.15 ± 0.28
Lymphocyte (x10$^{9/l}$)	Control	7.15 ±1.38	8.27 ± 0.63	8.19 ± 0.47[a]	5.90 ± 0.62[b]	7.22 ± 1.26[c]	5.87 ± 0.79[d]	5.07 ± 0.35[e]	5.70 ± 0.65[f]	4.13 ± 0.79
	Infected	8.02 ±0.24	9.15 ± 2.42	16.47 ± 1.78[a]	18.23 ± 0.93[b]	21.59 ± 0.92[c]	16.56 ± 1.08[d]	15.79 ± 0.59[e]	13.60 ± 0.77[f]	14.25 ± 2.12

- **values with the same superscript alphabets are significantly different with p<0.05**

APPENDIX 4

Mean (±SEM) of Biochemical Parameters of *E. coli* infected and control groups in commercial layers

Parameters		SAMPLING INTERVAL IN DAYS								
		0	**2**	**4**	**6**	**14**	**21**	**28**	**35**	**42**
	Control	1.14 ±0.03	1.13 ±0.03	1.11 ±0.02	1.067 ±0.04	1.05 ±0.02[a]	1.07 ±0.01[b]	1.17 ±0.02[c]	1.09 ±0.005[d]	1.07 ±0.007[e]
Phosphate (mmol/L)	Infected	1.11 ±0.02	1.14 ±0.04	1.09 ±0.03	1.16 ±0.07	1.64 ±0.12[a]	1.27 ±0.01[b]	1.41 ±0.03[c]	1.23 ±0.02[d]	1.23 ±0.03[e]
	Control	2.35 ±0.04	2.38 ±0.01	2.38 ±0.01	2.36 ±0.02	2.35 ±0.03[a]	2.37 ±0.02[b]	2.34 ±0.03[c]	2.37 ±0.02[d]	2.33 ±0.02[e]
Calcium (mmol/L)	Infected	2.41 ±0.05	2.37 ±0.10	2.33 ±0.018	2.24 ±0.05	1.61 ±0.11[a]	1.72 ±0.04[b]	1.68 ±0.07[c]	2.05 ±0.02[d]	2.02 ±0.08[e]
	Control	56.0 ±0.71	55.6 ±0.51	54.2 ±1.07	53.8 ±0.80[a]	52.8 ±1.24[b]	55.6 ±0.81[c]	55.2 ±1.02[d]	54.6 ±0.93[e]	55.6 ±1.03[f]
AST(IU/L)	Infected	55.8 ±0.66	54.8 ±1.02	55 ±1.00	59.2 ±1.20[a]	61.8 ±1.85[b]	64.6 ±1.17[c]	66.4 ±0.93[d]	63.6 ±1.44[e]	60.8 ±1.07[f]
	Control	63.2 ±1.16	63.6 ±1.50	66.5 ±4.62	60.4 ±0.93[a]	61.8 ±1.36[b]	61 ±1.05[c]	60 ±0.71[d]	61.4 ±0.81[e]	61.6 ±1.66[f]
ALT (IU/L)	Infected	63.0 ±1.30	63.5 ±1.40	64.0 ±1.38	70.2 ±1.20[a]	72.6 ±1.60[b]	74.4 ±2.11[c]	72.8 ±2.49[d]	73.6 ±1.96[e]	66.4 ±2.06[f]
	Control	7.88 ±0.61	7.68 ±0.39	7.32 ±0.57[a]	7.88 ±0.71[b]	7.08 ±0.31[c]	7.44 ±0.35[d]	7 ±0.55[e]	7.24 ±0.62[f]	7.08 ±0.31[g]
Total Protein	Infected	7.84 ± 0.37	7.12 ±0.31	6.04 ±0.32[a]	5.8 ±0.33[b]	5.52 ±0.43[c]	5.28 ±0.54[d]	5.2 ±0.21[e]	5.12 ±0.28[f]	5.56 ±0.49[g]
	Control	373 ±2.55	364 ±4.30	362 ±6.82	365 ±5.00	378 ±8.75[a]	378 ±3.74[b]	364 ±4.30[c]	371 ±3.32[d]	365 ±3.54[e]
Oestrogen (pg/ml)	Infected	377.2 ±5.24	364 ±5.30	363 ±2.60	357 ±4.90	347 ±2.60[a]	338 ±6.40[b]	332.6 ±5.40[c]	334 ±2.90[d]	340 ±7.10[e]
	Control	130.08 ±2.87	132.28 ±1.86	132.2 ±1.77[a]	133.2 ±2.03[b]	133.2 ±1.43[c]	133.8 ±1.83[d]	134.8 ±1.36[e]	135.2 ±1.07[f]	134.4 ±1.78[g]
Progesterone (pg/ml)	Infected	131.9 ±2.10	133.3 ±2.42	117.1 ±2.98[a]	109 ±1.79[b]	103 ±2.95[c]	96.2 ±3.09[d]	94.8 ±1.98[e]	97.6 ±2.66[f]	104.4 ±2.54[g]
	Control	25.36 ±0.21	25.06 ±0.34	24.6 ±0.29	24.2 ±0.66	24.90 ±0.29[a]	24.5 ±0.32[b]	24.8 ±0.41[c]	24.5 ±0.42[d]	23.9 ±0.90
PTH (pg/ml)	Infected	25.52 ±0.24	25.30 ±0.24	24.30 ±0.20	24.10 ±0.56	27.40 ±0.79[a]	31.04 ±0.80[b]	27.40 ±0.58[c]	28.50 ±0.55[d]	23.50 ±0.63

- **values with the same superscript alphabets are significantly different with $p<0.05$**