IMMUNE RESPONSE OF BROILER CHICKENS TO CAECAL COCCIDIOSIS USING EXO AND ENDOGENOUS STAGES OF *Eimeria tenella*

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IMMUNE RESPONSE OF BROILER CHICKENS TO CAECAL COCCIDIOSIS USING EXO AND ENDOGENOUS STAGES OF *Eimeria tenella*

BY

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A THESIS SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES AHMADU BELLO UNIVERSITY ZARIA, IN PARTIAL FULFILLMENT FOR THE AWARD OF DOCTOR OF PHILOSOPHY IN VETERINARY PARASITOLOGY

DEPARTMENT OF VETERINARY PARASITOLOGY AND ENTOMOLOGY,

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ZARIA ,NIGERIA

JANUARY, 2017

DECLARATION

I declare that the work in this Thesis entitled "**Immune Response of Broiler Chickens to Caecal Coccidiosis Using Exo and Endogenous Stages of *Eimeria tenella***" has been performed by me in the Department of Veterinary Parasitology and Entomology. The information derived from literature has been duly acknowledged in the text and a list of references provided. No part of this Thesis was previously presented for another degree or diploma at this or any other Institution.

Paul Davou KAZE _____ _____

Signature Date

CERTIFICATION

This Thesis entitled "**IMMUNE RESPONSE OF BROILER CHICKENS TO CAECAL COCCIDIOSIS USING EXO AND ENDOGENOUS STAGES OF *EIMERIA TENELLA***" by Paul Davou, KAZE meets the regulations governing the award of the degree of Doctor of Philosophy of Ahmadu Bello University, Zaria, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This Thesis is dedicated to Almighty God and my entire family

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ABSTRACT

The aim of this study was to determine the immune response of broiler chickens to *Eimeria tenella* developmental stages Four hundred broilers divided into six groups (n=40) were used for the study. Each group was subdivided into two (n=20) as treated and non-treated and infected with different developmental stges of *Eimeria tenella* (local isolate). The molecular identification of the local *Eimeria tenella* isolate identity was done through polymerase chain reaction (PCR) amplification of the genomic deoxyribonucleic acid (DNA). Clinical signs, gross caecal lesions, humoral and cellular-mediated immune responses were determined in the infected broiler chickens with *Eimeria tenella* developmental stages. The faeces were processed using simple floatation technique and observed at x 10 and x 40 objectives of the Neiss microscope. Oocysts isolated from the caeca of birds naturally infected in Jos, Nigeria with the local strain were used to obtain the different developmental stages either *in vitr*o or *in vivo* using bovine monocytes (schizonts), embryonated chicken eggs (gamatocytes) and two weeks old broilers (merozoites). To study the immune response elicited during the primary and secondary infection, each developmental stage was used to infect a group of two, three and half weeks old broilers, twenty of which were treated with the recommended dose of amprolium (250 mg/l (0.025%) for 5 days at the appearance of clinical signs. At the tertiary infection, all the experimental birds except the control group of forty birds were orally infecteded with 10^5 sporulated oocysts of known characterized virulent *Eimeria tenella* strain. The mean oocysts output or count was 37.07 x 10^6 in the infected birds non-treated than 25.65 x 10^6 in the treated groups, although there was a gradual reduction (groups II – 8.36 x 10^6 – 7.84 x 10^6 – 5.10 x 10^6; III - - 6.58 x 10^6 – 4.83 x 10^6; IV – 7.18 x 10^6 – 7.00 x 10^6 – 3.83 x 10^6; V – 6.59 x 10^6 – 5.87 x 10^6 – 4.20 x 10^6) in oocyst count from primary-secondary-

tertiary infections except group I (control). There was a significant difference in oocyst output between the groups (II and IV) ($p<0.05$). Antibodies (IgG or IgY) titre values were higher in broilers sera infected with sporulated oocyst (0.265 ± 0.010, 0.282 ± 0.005; 0.305 ± 0.002, 0.316 ± 0.010 and 0.252 ± 0.002, 0.281 ± 0.010) and merozoites (0.177 ± 0.001, 0.186 ± 0.003; 0.135± 0.010, 0.141 ± 0.002 and 0.069 ± 0.004, 0.139 ± 0.005) reaching a peak on day 10 of post primary and secondary infections and day 5 post tertiary infection in sera of broilers (treated and non- treated). At tertiary infection, antibodies increases at day 5, 7, 11 and 14 indicating that antibodies increases in broilers infected with the invasive or zoite stages, (sporozoite and merozoite) of the parasite.. There was a significant difference in the antibody output between the sera of the broiler groups ($p<0.05$). The study reveals the proliferation of cytokines in treated and non- treated broilers consisting of IFN- γ, IL-1, IL-2, IL-4, IL-6, TNF and TGF. The CD_4 lymphocyte count in the treated and non- treated broilers orally administered with various developmental stages of the parasite reached a peak at day 10 ((groups I – 198.0 x 10^3 µl, 165.3 x 10^3 µl; 200.0 x 10^3 µl, 156 x 10^3 µl and 196.7 x 10^3 µl, 173.3 x 10^3 µl ; II – 199.0 x 10^3 µl, 186.0 x 10^3 µl ; 197.0 x 10^3 µl, 192.7 x 10^3 µl and 200.0 x 10^3 µl, 194 x 10^3 µl; III – 198 x 10^3 µl, 153.3 x 10^3 µl ; 200.0 x 10^3 µ,l 160.0 x 10^3 µl and 188.7 x 10^3 µl, 166.7 x 10^3 µl ; IV – 193.3 x 10^3 µl, 183 x 10^3 µl; 198.7 x 10^3 µl, 183.3 x 10^3 µl and 190 x 10^3 µl , 188.0 x 10^3 µl ; V – 200.0 x 1 0^3 µl, 198.0 x 10^3 µl ; 187.3 x 10^3 µl , 174 x 10^3 µl and 188.7 x 10^3 µl, 175.3 x 10^3 µl respectively) at primary and secondary infections and day 24 at tertiary infection. There was significant difference in the CD_4 cell count between groups of the infected broiler chickens ($p<0.05$). Caecal lesions were observed to gradually reduce from primary-secondary-tertiary infections. The lesions were significantly different

between in groups (II and IV) ($p<0.05$). Oocyst output and caecal lesions were absent group VI (control). The current study observed a relationship between the different developmental stages of the parasite and immune responses (humoral and lymphocytes responses). The study also observed that broilers with high oocyst output had high antibody production, CD_4 lymphocytes count and high levels of cytokines. Thus, the sporozoites and merozoites are the invasive stages that initiate infection of host cells and probably stimulation of the immune response and may be possible vaccine candidate against avian coccidiosis.

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LIST OF ABBREVIATIONS/ACRONYMS

AA	Aracchidonic acid
ABU	Ahmadu Bello University
ACD	Acid-citrate-dextrose
ALA	Alpha-linolenic acid
AMV	Avian Myeloblastosis Virus
ANOVA	Analysis of variance
BALT	Bronchial-associated lymphoid tissue
BCIP	5-bromo-4-chloro-3-indodyphosphate
Bp	Base pair
BSA	Bovine serum albumin
CD	Cluster of Differentiation
cDNA	Complementary Deoxyribonucleic Acid
CFMV	Consello Federal de Medicina Veternaria
DHA	Dacosahexaenoic acid
DMEM	Dulbecco Modified Eagle Medium
dNTP	Deoxynucleotide Triphosphates
EC	European Countries
ECWA	Evengelical Church of West Africa
ED	Embryonic Development
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme Linked Immuno-sorbent Assay
ELISPOT	Enzyme Linked Immuno-Sorbent Assay
EMF	Electromagnetic Fields
EPA	Eccosapentaenoic acid
EU	European Union
FAO	Food and Agriculture Organization
FBS	Foetal bovine serum
FITC	Fluorescin isothiocyanate
GALT	Gut associated lymphoid tissue
GIT	Gastrointestinal tract

HBSS	Hank's Balanced Salt Solution
HS	Horse serum
IBD	Infectious bursal disease
IEL	Intraepitheliel Lymphocytes
IFN-γ	Interferon gamma
IL	Interleukins
ITS	Internal Transcribe Sequence
MFR	Member of the Federal Republic
MOS	Mannanoligosaccharides
NBT	Nitro-blue tetrazolium
NK	Natural killers
NT	Non-Treated
NVRI	National Veterinary Research Institute
O.D	Optical Density
PADP	Plateau Agricultural Development Programme
PBL	Peripheral Blood Lymphocytes
PBS	Phosphate buffered saline
PBST	Phosphate buffer saline tween
PCKC	Primary Chick Kidney Cells
PRF	Polyphenol-Rich Fraction
PUFA	Polyunsaturated Fatty Acids
rDNA	Ribosomal Deoxyribonucleic acid
RPMI	Rosewell Park Memorial Institute medium
rRNA	Ribosomal Ribonucleic acid
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
S/T	Saline/Tween
SAGs	Surface antigens
SCE	Sugar Cane Extract
T	Treated
TGF	Transforming Growth Factor
TNF	Tumor Necrotic Factor

TETFUND	Tertiary Education Trust Fund
UK	United Kingdom
US	United States
WHO	World Health Organisation

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Avian coccidiosis is caused by intracellular protozoans parasites belonging to seven species of *Eimeria. Eimeria tenella* is the most virulent causing severe heamorrhagic enteritis by infection of the epithelium and submucosa of the caeca and eventually death in infected chickens (Mehlhorn, 2005). Lawal *et al*. (2008) showed that the infection can occur in both local and exotic birds with the former serving mainly as reservoir hosts. The parasite development cause diarrhoea, morbidity and mortality, and has serious economic impact in the poultry industry (Takagi *et al*., 2006; Gyorke *et al.*, 2013). Coocidiosis causes annual losses of US $ 2.4 billion to the poultry industry worldwide (Shirly *et al.*, 2005) in both the layer and broiler industries (Chandrakesan *et al*., 2009).

Conventional disease control strategies depend on vaccination and proplylactic use of anticoccidial drugs. However, resistance against anticoccidial compounds is widely spread and coccidiostats as feed additives was banned in Europe by the year 2012 (Regulation (EC) No 1831/2003 of the European parliament and of the council of 22 September, 2003 on additives for use in animal nutrition). *Eimeria* infection promotes antibody and cell-mediated immune responses, and cellular immunity mediated by various cell populations, including lymphocytes, natural killer (NK) cells and macrophages plays a major role in disease resistance (Lillehoj and Choi, 1998). There is increase evidence of CD_4^+ and intraepithelial lymphocyte (IEL) involvement during a primary infection, while T-cell receptor α-and β-chain-positive CD_8^+ IEL play a key role in secondary infection (Lillehoj, 1998). The low level of homology between chicken genes and their mammalian

counterparts has made it difficult to discover immunologically relevant chicken genes. However, there have been increasing numbers of chicken gene sequences appearing in the data bases due to the emergence of chicken genome projects. Among the cytokines cloned, one can find gene coding for interleukins (interleukin -1β (IL-1β), (Weining *et al*., 1998), IL-2 (Sundick *et al*., 1997) and interferons (alpha/beta interferon [IFN- α/β] (Sekellick *et al*., 1994) and IFN- γ (Digby and Lowenthal, 1995) and also for a macrophage growth (Leutz *et al.*, 1989) and three isofornes of transforming growth factor β (TGF-β) (Jakowlew *et al*; 1990). In addition, several members of the chemokine family, have recently been cloned: C chemokines cc chemokines (macrophage inflammatory protein 1^{β} (MIP-1^{β}) (Hughes *et al*;, 1999) and K203 (Sick *et al*., 2000) and C X C chemokines (k60 (Sick *et al*., 2000) and IL-8 (Kaiser *et al*.., 1999). A number of receptors have also been identified including the IL-1 receptor (IL-IR) (Guida *et al*., 1992) and a putative chemokine receptor (chem.-R) (Gupta *et al*., 1998).

However, the analysis by reverse transcription – polymerase chain reaction (RT-PCR) of the expression of an available panel of genes will provide initial clues about the development of immune response to *Eimeria* infection. In this study, we intend to analyse the local immune response of broiler chickens to *Eimeria tenella* commonly found in poultry farms in Jos, Nigeria by PCR, lymphoproliferation assay or non radioactive assay, Enzme linked-immunosorbent assay (ELISA) and flow cytometric analysis.

1.2 Statement of the Problem

The creation of states in Nigeria and increase human population and activities within the states to earn money for living and increase in population has led to establishment of several poultry farms to meet the increasing demands for animal protein in terms of meat and eggs (Adegoye *et al*., 1988; FAO, 2006). Chickens also fulfill an array of other functions such as pest control, provision of manure and sacrifice in special festivals. However, one of the major constraints to production of chickens is coccidiosis (Sani *et al*. 1987; Majoro, 1993; Biu *et al*., 2006).

In Nigeria where intensive poultry farming is less developed, the rising cost of poultry feeds, problems of drug residues, lack of new anticoccidial products and resistance to diseases are major problems militating against the poultry industry (Ogbe *et al*., 2008; Hafez, 2008). Coccidiosis is a serious disease which causes heavy economic loss. It has remained the most important poultry disease in Nigeria (Obasi *et al*., 2006). The macroscopic lesions in the digestive tract predispose the infected birds to many gastrointestinal bacterial poultry diseases such as clostridiosis, salmonellosis and collibacillosis (Lanckriert *et al*., 2010). Mua'zua *et al*. (2008) reported a prevalence rates of 52.9% and 36.7% coccidial infection among the adult and younger birds in Vom, Plateau State, Nigeria.

Although the exact losses due to coccidiosis in Nigeria are not known due to lack of statistical indices but they could be in the region of millions of naira. The annual worldwide cost is estimated at about US$2.4 Billion (Shirley *et al*,, 2005).

Problems associated with antigenic variation of field strains and the cost of producing multiple-species live vaccines post limits to the current vaccination approaches (Constantinoiu *et al*., 2008). The cost involved in the production of recombinant vaccines (proteins or DNA), the difficulty in identifying the antigens or genes that can elicit coccidia-specific protective immunity and the devise of the most efficient method of delivering these recombinant vaccines to the bird immune system are additional obstacles (Bedran and Lukesova, 2006). Besides, there are also the increasing incidence of drug-resistant strains and escalating public anxiety over chemical residues in meat and eggs as well as the complexities of the host immune system and parasite life cycle (Bal, 2009).

1.3 Justification of the Study

Coccidiosis is a protozoan disease of poultry commonly occurring under intensive management system (Biu *et al*., 2006). This makes coccidiosis one of the major health problems of chickens in Nigeria. An outbreak of coccidiosis in a poultry flock has a very high negative and economic impact. The disease is an important component of poultry diseases, and it is responsible for high morbidity, mortality and reduced market value of affected birds, and sometimes leading to culling or delayed slaughter time. Coccidiosis leads to destruction of the integrity of the intestinal mucosae and interference with nutrient absorption, causing diarrhoea and increase in medication costs. All these setbacks lead to huge losses for the producer. Treatment and control of the disease are beset with several problems prominent of which is the poor understanding of the immune response. Another factor is the increasing incidence of drug resistance in field strains of *Eimeria*.

Knowledge of the immune response to the different stages of *E. tenella* will give an insight on the possibility of control of the disease through vaccine production, which will ultimately lead to increase in productivity. Furthermore, due to health awareness, there is increasing concern regarding drug residues in poultry products and growing pressure from Government and consumer on the production of drug-free poultry products (Williams, 2002).

Consequently, the use of vaccines has become more desirable than ever before. Epidemiological studies have established the economic importance of coccidiosis as a major parasitic disease of poultry in Nigeria (Chapman, 2008).

1.4 Aim of the Study

To determine the immune response of broilers to different developmental stages of *Eimeria tenella*.

1.5 Objectives of the Study

The objectives of the study are to:-

1. Monitor the clinical signs in the experimental infection (oral) of birds with different *Eimeria tenella* developmental stages.
2. Determine the gross caecal lesions in broiler chickens cause by infection with different stages of *Eimeria tenella*.
3. Determine the immune responses to the various stages of *Eimeria tenella* in the orally infected broiler chickens by measurement of immune bodies.

4. Determine the conferment of immunity in the broiler chickens infected with *Eimeria tenella* developmental stages.

1.6 Research Questions

1. Do exogenous (unsporulated and sporulated oocysts) and endogenous stages (schizonts, merozoites and gametocytes) of *Eimeria tenella* induce clinical signs in the experimentally infected birds?
2. Do exogenous (unsporulated and sporulated oocysts) and endogenous stages (schizonts, merozoites and gametocytes) of *Eimeria tenella* induce gross caecal lesions in the experimentally infected birds?
3. Do exogenous (unsporulated and sporulated oocysts) and endogenous stages (schizonts, merozoites and gametocytes) of *Eimeria tenella* induce immune responses in the experimentally infected birds?

CHAPTER TWO

LITERATURE REVIEW

2.1 Introduction

Coccidiosis is an infectious disease caused by protozoan parasite of the Phylum: Apicomplexa, Family: *Eimeriidae* and Genus *Eimeria* as (Tyzzer, 1932 as cited by Hussain, 2010). It is a disease of universal importance in poultry production. Coccidia are distributed worldwide in poultry, game birds reared in captivity, and wild birds. The disease may strike any type of poultry in any type of facility. The parasite multiplies in the intestinal tract and causes tissue damage, resulting in diminished feed intake and nutrient absorption, reduced body-weight gain, dehydration, blood loss, and increased susceptibility to other diseases (McDougald, 2003). The induced tissue damage and change in intestinal function may allow colonization by various harmful bacteria, such as *Clostridium perfringens*, leading to necrotic enteritis (Maxey and Page, 1977). Caecal coccidiosis caused by *Eimeria tenella* may contribute to an increased severity of blackhead disease in chickens (McDougald and Hu, 2001). Coccidiosis remains one of the most expensive and common diseases of poultry production in spite of advances in chemotherapy, management, nutrition, and genetics. It costs chickens producers worldwide at least 3 billions $US annually (Dalloul and Lillehoj, 2006).

2.2 Aetiology

Coccidia of chickens have been the subject of intense study and there are more documented details in their life cycle, physiology, pathology, and prophylactic and therapeutic control than on those of similar other parasites Allen and Fetterer (2002) and McDougald (2003). There are many *Eimeria* species that can infect chickens, but there are seven species of *Eimeria* that parasitize chickens (*Gallus gallus*). These species are *E. acervulina, E. brunetti, E. maxima, E. mitis, E. necatrix, E. praecox, E. tenella* and they occur in domesticated fowls (Davis *et al.*, 1973; Long *et al.,* 1976*;* Vermeulen (1998)

Williams, (1998) Allen and Fetterer (2002) and McDougald (2003) summarized the criteria that are important in the identification of species as follows:

1. location and macroscopic appearance of the lesion in the intestine .
2. oocyst size, shape, and colour.
3. size of schizonts and merozoites.
4. minimum prepatent period in experimental infection.
5. location of the parasite in the tissues (type of cell parasitized).
6. immunogenicity against reference strain.
7. stage of the life cycle that produces most tissue damage
8. molecular and biological approach: electrophoresis of metabolic enzymes PCR (Tsuji *et al*., 1997; Shirley, 2000).

Both clinically infected and recovered birds shed oocysts in their droppings, which contaminate feed, dust, water, litter and soil.Pathogenecity is influenced by host genetics, nutritional factors, concurrent diseases, age of the host and species of the coccidium. Protective immunity usually develops in response to moderate and continuing infection.True age-immunity does not occur, but older birds are usually more resistant than younger ones due to earlier exposure to infection.

2.3 Coccidiosis and Intestinal Microflora

Extensive reviews (Fuller, 1989; Ewing and Cole, 1994) on the role of intestinal microflora on animal performance support Schaedler's statement. Many studies have documented the important role of intestinal microflora in promoting the incidence and the severity of coccidiosis (Fuller 1989; Ewing and Cole, 1994) . It is confirmed that caecal coccidiosis does not occur in chickens fed a diet designed to depress putrefying bacteria, while it occurs in chickens fed a diet designed to promote proliferation of anaerobic putrefying bacteria in the intestine. It has been shown that clinical signs and mortality did not occur in bacteria-free chickens infected with surface-sterilized *Eimeria tenella* oocysts, but while chickens with two or more species of normal intestinal bacteria developed more severe lesions of coccidiosis and mortality than do their bacteria-free counterparts (Radhakrishnan and Bradley, 1972; Visco and Burns, 1972 a and b). The indigenous bacteria aid in the development of large numbers of the endogenous stages of *E. tenella* and typical caecal coccidiosis in chickens (Bradley and Radhakrishnan, 1973).

During the course of caecal coccidiosis, the growth of C*lostridium perfringens* and coliforms, especially *E. coli*, is stimulated and the growth of *Lactobacillus* species. is suppressed. Turk and LittleJohn (1987) studied the effects of *E. acervulina, E. necatrix, E. brunetti,* and *E. tenella* on the composition of the gut microflora. They reported that the number of the faecal anaerobes was increased on the 6^{th} day of *E. acervulina* infection, on the 3^{rd}, 6^{th}, 7^{th}, and 14^{th} days of *E. necatrix* infection, and on the 3^{rd} and 6^{th} days of *E. brunetti* infection. Turk and LittleJohn (1987) also reported an increase in faecal *Lactobacilli* in *E. necatrix*-infected birds during the period of 16 to 18 days post infection, and on the 8^{th} day in *E. necatrix* infection. However, the faecal coliforms increased in all

infections on the 6th day. The authors related the observed changes in the microflora population to the changes in the residual nutrients found in the gut, resulting from malabsorption of nutrients by the host due to attack by the parasite.,

2.4 Taxonomy of *Eimeria tenella*

2.4.1 Classification

Kingdom: Protista

Phylum: Apicomplexa

Class: Conoidasida

Order: Eucoccidiorida

Family: Eimeriidae

Genus: *Eimeria*

Species: *Eimeria tenella*

Binomial name*: Eimeria*

Mnemonic: EIMTE

Taxon identifier: 5802

Common name: Coccidian parasite

E. tenella and related strains

Strain name

1 E. tenella Beijing

2 E. tenella H

3 E. tenella Houghton

4 E. tenella LS18

5 E. tenella PAPt38

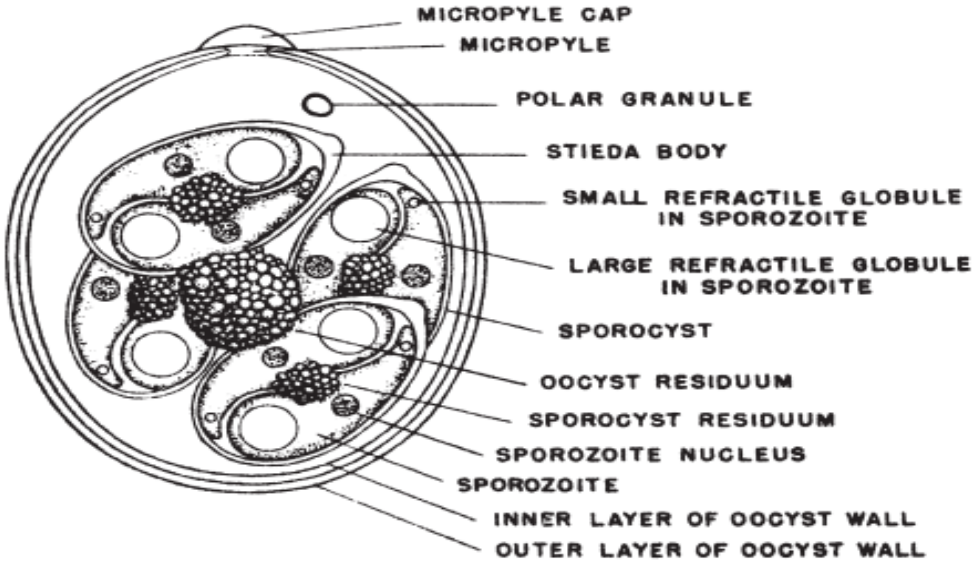
6 E. tenella Penn State

7 E. tenella Weybridge

8 E. tenella Wisconsin, Wis

2.5 Morphology of *Eimeria* Species

Eimeria spp. are frequently described from the morphology of the oocyst, a thick-walled zygote shed in faecal material by the infected host. An average of *Eimeria. tenella* oocyst dimensions are 23x19micrometer (μm) in length and 17x20 micrometer (μm) in width. Oocysts are enclosed in a thick outer shell and consist of a single cell that begins the process of sporulation to yield the infective stage in about 48 hours. Infective oocyst contains four sporocysts (Figure 2.1) which in turn contain two sporozoites (Saif *et al.*, 2003). A membrane consists of three layers (one layer of lipoprotein between two layers of protein). *Eimeria* spp. secretes enzymes to destroy host cell membrane and get oxygen resulting from digested nutrient.



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Figure 2.1 : Diagram of sporulated oocyst of *Eimeria* species.
Source: Zander and Mallisson (1991).

2.6 Life Cycle of *Eimeria tenella*

Infection occurs when a susceptible chicken ingests a sporulated oocyst from its environment. The sporulated oocyst contains four sporocysts, each sporocyst contains two sporozoites. The sporozoites are released by mechanical and biochemical action in the digestive tract of the chicken (Reid and Yoder, 1978). Sporozoites escape from the sporocysts and oocysts, but the factors contributing to excystation have not been definitely established, pancreatic juice, in particular trypsin, is one of the factors responsible for excystation (Zander and Mallinson, 1991). . Escape of sporozoites through any available fracture in the cyst occurs 5-10 minutes after the cyst wall has been placed in a trypsin solution, maintained at 37° C. Hydrogen-ion concentration, bile and buffers were also found to be important factors in excystation of various species of Coccidia (Ghittur, 1971). The effects of pH, buffers, bile and bile acids on the excystation of sporozoites of various *Eimeria* species including *E. tenella*, found no excystation when any of the bile acids, bovine or chicken bile was used alone without trypsin (Reid and Yoder, 1978) . Therefore the release of large numbers of sporozoites into the digestive tract of chickens requires the wall of the oocyst to be broken. The walls of many sporulated oocysts expelled through faeces has been shown to be structurally changed (Ghittur, 1971).

The liberated sporozoites invade epithelial cells in a specific zone of the intestine or caeca depending on the species. Upon entering the host cell, the sporozoite transforms in 12 to 48 hours to a feeding stage called a trophozoite. The trophozoite begins to enlarge, and the parasite nucleus divides by a process of asexual multiple divisions known as schizogony (merogony). At this point, the parasite stage is referred to as a schizont or meront (Ghittur, 1971). The small parasitic stages forming within the schizont are called merozoites. The

schizont ruptures when matured on the third day, releasing the merozoites. Most of these invade other epithelial cells to repeat the process of development through the trophozoite and schizogonous stages. The merozoites from the second schizogonous cycle again penetrate the epithelial cell of the host. Some or all may go through a third schizogonous cycle, depending on the species, before formation of male (microgametocytes) or female (macrogametocytes). The male gametocyte matures and ruptures, releasing a large number of minute biflagellate microgametes and fertilized the female gametocyte. The macrogametocyte grows to form a macrogamete (Zander and Mallinson, 1991). A thickened wall forms around the macrogamete, forming a zygote when the macrogamete is fertilized by a microgamete. This stage is the young (immature oocyst) unsporlated oocysts. The prepatent period varies with each species depending on the time required for each schizogonous cycle and the number of cycles. The oocyst ruptures the host cell when mature and passes out of the bird in the droppings. Under suitable environmental conditions, four sporocysts each containing two sporozoites, are formed within the oocyst after about 24 hours (Zander and Mallinson, 1991). This parasite develops in the cells of the caeca. Infections may be characterized by the presence of blood in the droppings and by high morbidity and mortality which can be significant (Micheal, 1999).

2.7 *In vitro* (Experimental) Cultivation of *Eimeria tenella*

Important prerequisites for the successful *in vitro* cultivation of *Eimeria tenella* in primary chick kidney cells (PCKC) are optimal conditions for the controlled growth of PCKC and the Coccidia parasite, i.e. the use of suitable nutrient media, concentrations and quality of fetal calve serum as well as the production of ultrapure sporozoite suspensions. It has been possible to film the complete life cycle of *E. tenella in vitro*. Motion pictures of all

endogenous and exogenous developmental stages of the parasite demonstrated the invasion of sporozoites into host cells and their further development to schizonts of the first, second and third generation by multiple asexual reproductions (schizogony), the maturation of female and male gamonts to respective gametes, and finally the formation of zygotes (Woltgang *et al.*, 1999).

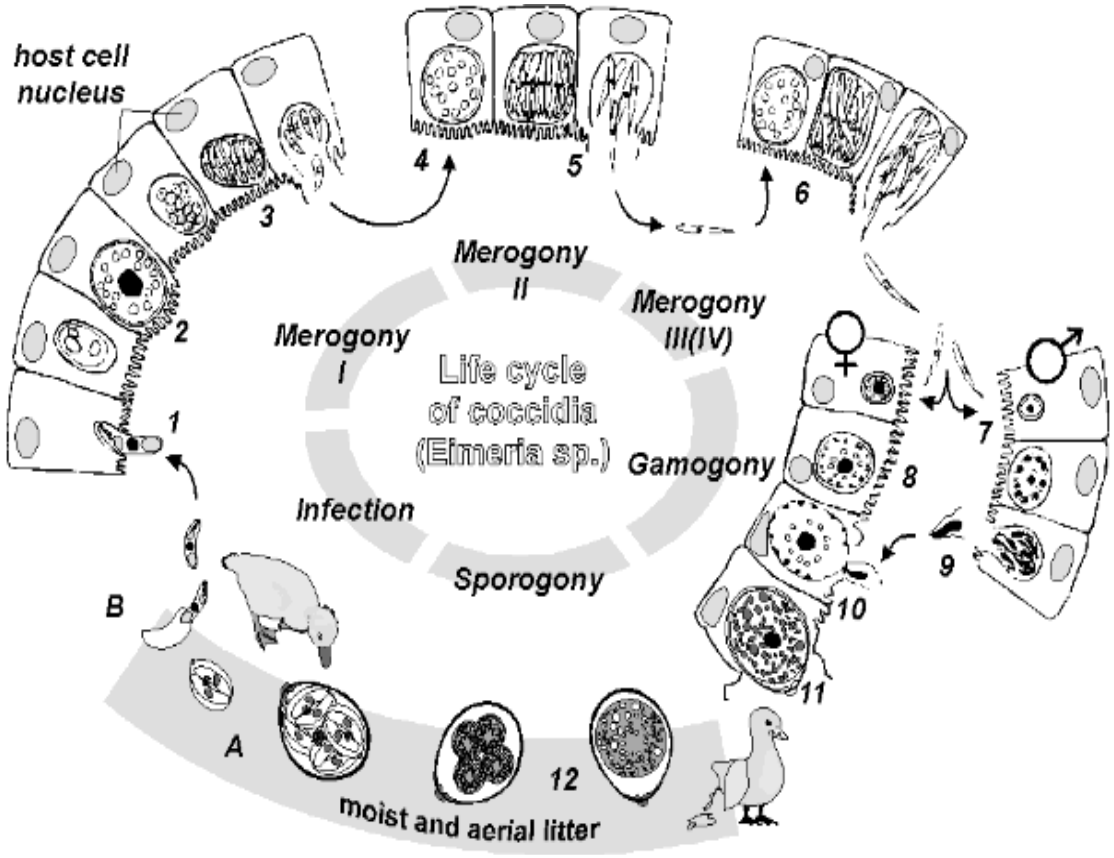


Figure 2.2 : Life cycle of *Eimeria tenella*. Source: Anne (2006).

2.8 Overview of Chicken Coccidiosis

Poultry are kept in backyards or commercial production systems in most areas of the world. Compared to a number of other livestock species, fewer social and religious taboos are related to the production, marketing, and consumption of poultry products (Graat *et al.*, 1996). . For these reasons, poultry products have become one of the most important protein sources for people throughout the world (Mashishi, 2002). The total number of poultry in the world has been estimated by the Food and Agriculture Organization of the United Nations (FAO) to be 14,718 million, with 1,125 million distributed throughout the Africa1,520 million in South America and 6,752 million in Asia, 93 million in Oceania,3,384 million in North America and 1,844 million in Europe (Anders and Jorgen, 1998). A disease resulting from parasitism depends on the number, type, and virulence of the parasite, the route of entry to the body, the defense status and capabilities of the host. The latter depends partly on the host's prior disease encounters (e.g. infectious bursal disease, IBD), nutritional status, and genetic ability to organize resistance mechanisms, environmental stresses and the kind and timing of counter measures employed such has drugs or changes of environment (Saif *et al.*, 2003). Parasites can occur inside the chicken (internal parasites e.g. worms) or on the outside of the chicken. Internal parasites can be classified into several types based on their body types, life cycle, and damage to their hosts (Conway and Mckenzie, 2007).

Internal parasites of poultry include roundworms or Nematodes, tapeworms or Cestodes, flukes or Trematodes, and Protozoa which include Coccidia, *Cryptosporidia , Histomonas* spp. (Blackhead), *Trichomonas* spp. and other blood and tissue protozoa (Gray, 2002). Coccidiosis remains one of the major disease problems of poultry in spite of advances

made in prevention and control through chemotherapy, management and nutrition (Graat *et al.,* 1996). Coccidia of the genus *Eimeria* are predominately host-specific, each species occuring in a single host species or a group of closely related hosts. Infection by *Eimeria* oocysts should be in sufficient numbers to produce clinical manifestations of disease that is named coccidiosis. Differential identification of each species is dependent upon the following characteristics; zone of intestine parasitized, gross appearance of the lesion, oocyst morphology, minimum sporulation time, minimum prepatent time, schizont size, location of parasite in the host intestinal epithelium and Cross immunization tests (Conway and Mckenzie, 2007). *Eimeria tenella* and *E. necatrix* are the most pathogenic species (Soulsby, 1982). *E. tenella* infections are found only in the caeca and it is called caecal coccidiosis. In addition it can be recognized by accumulation of blood in the caeca and by bloody droppings. Caecal cores, which are accumulations of clotted blood, tissue debris, and oocysts, may be found in birds surviving the acute stage (Bafundo and Donovan, 1988; Allam, 1989). Caecal coccidiosis causes decreasing of production and economic losses, which can be significant (Micheal, 1999).

2.9 Transmission of Avian Coccidiosis

Chickens become infected with *Eimeria species* by ingesting infective oocysts (eggs) from litter, soil and contaminated feed and water. The infected birds excrete oocysts into their faeces and are a source of infection for other birds as *Eimeria* spp can survive for long periods in infected birds and the environment (Khan *et al.,* 2006). The oocysts in faeces become infective through the process of sporulation in about two days (Jeurissen *et al.,* 1996). Birds in the same flock may ingest the oocysts through litter pecking or the contamination of feed or water. Although no natural intermediate hosts exist for the

Eimeria species many different animals, insects, contaminated equipment, mice, wild birds, and dust can spread oocysts mechanically. Oocysts generally are considered resistant to environmental extremes and to disinfectants, although survival time varies with conditions. Oocysts may survive, for many weeks in soil, but survival in poultry litter are limited to a few days because of the heat and ammonia released by composting and the action of molds and bacteria (Khan *et al*., 2006). Viable oocysts have been reported from the dust inside and outside broiler houses, as well as from insects in poultry litter.

Oocysts are quickly killed by exposure to extreme temperatures or drying. Exposure to 55°C or freezing kills oocysts very quickly. Even 37°C kills oocysts when continued for 2-3 days. Sporozoites and sporocysts can be frozen in liquid nitrogen with appropriate cryopreservation technique, but oocysts cannot be adequately infiltrated with cryoprotectants to effect survival.The darkling beetle, common in broiler litter, is a mechanical carrier of oocysts. Transmission from one farm to another is facilitated by movement of personnel and equipment between farms and by the migration of wild birds, which may mechanically spread the oocysts. New farms may remain free of Coccidia for most of the first grow out of chickens until the introduction of Coccidia to a completely susceptible flock. Such outbreaks, often more severe than those experienced in older farms and are often called the new house syndrome.

Threat of coccidiosis is less during hot dry weather and greater in cooler damp weather (Saif *et al.,* 2003). Recovered chickens shed oocysts representing a problem in multi-age operations (Sherif *et al.,* 2008). Factors contributing to outbreaks of clinical coccidiosis include;litter moisture content exceeding 30% due to ingress of rain or leaking water,

immunosuppression (Marek's, IBD and Mycotoxins diseases), suboptimal inclusion of anticoccidials or incomplete distribution (poor mixing) in feed and environmental and managemental stress such as overstocking, inoperative feeding systems, inadequate ventilation (Simon, 2005).The cost of anticoccidial feed additives and treatment is estimated to exceed 400 million US$ annually in all poultry producing areas of the world (Simon, 2005). Hence, particular vaccines may be designed for rearing standard broilers for up to about 6 weeks or for breeding stock (Williams, 1998). Coccidia have been found where ever poultry are raised.

The spread of this parasitic disease is enhanced by poor bio-safety and management practices. While the *Eimeria* species that are known to infect chickens, typically a poultry facility will contain only 1-3 species of *Eimeria* that are known to infect chickens at a time (Saif *et al*., 2003). In Nigeria, the species *E. acervulina, E. maxima, E. necatrix* and *E. tenella* are found most often. However, reports of increased incidence of *E. mitis* and *E. praecox* have been surfacing. There is evidence that protection against the major species of Coccidia will allow for the emergence of minor species in a poultry operation. Thus, vaccines using oocyst based or subunit must provide protection against other species that are pathogenic for chickens. The disease is often diagnosed in birds brought to diagnostic laboratories but the vast majority of cases are diagnosed in the field and handled by poultry service personnel.

2.10 Prevalence, Epidemiology and Economic Impact of Coccidiosis

Coccidiosis remains one of the major disease problems of poultry in spite of advances made in prevention and control through chemotherapy, management and nutrition. *Eimeria*

tenella and *E. necatrix* are the most pathogenic species (Getachew *et al.*, 2008). The disease causes high mortality, morbidity and adverse effects on the growth of infected birds (Anjum, 1990). The incidence of coccidiosis in commercial poultry has increased due to higher stocking densities and intensive husbandry practices (Ruzica *et al.*, 2005). Coccidiosis occurs worldwide and is a major cause of mortality and suboptimal growth and feed conversion efficiency in immature flocks unless appropriate preventive measures are implemented. Medication exceeds 90 US$ million in the US. Surveys in North and South America revealed Coccidia present in almost all broiler farms (Saif *et al.*, 2003). Very high percentages of positive flocks were also, reported from Europe Northern Argentina prevalence was 14% in 1986. Presence of all known *Eimeria* spp. recorded in France 1996, Sweden 1990 and Korea 1984. Several studies in Japan leave little doubt of the presence of all species in country.

In Nigeria, Mu ʾazu *et al*. (2008) reported a prevalence rate of coccidial infection among young birds as 52.9% and among the adult birds as 36.7% respectively in Vom, Plateau State. In china the prevalence of the infection rate of identified *Eimeria* spp. was 90%, 88%, 72%, 68%, 60%, 26%, and 8% for *E. tenella*, *E. praecox*, *E. acervulina*, *E. maxima*, *E. mitis*, *E. necatrix*, and *E. brunetti*, respectively (Sun *et al*., 2009). Coccidiosis is one of the most important and common diseases that affect poultry, it results in a great economic loss all over the world**,** (Nematollahi *et al.*, 2009). Worldwide, coccidiosis causes more than 3 US$ billion in economic damages each year. Prevention of the spread of the disease among broilers is primarily based on hygiene measures and adding anti-coccidial drugs to feed or drinking water (Herman, 2010). The impact of disease on animal agriculture is typically assessed in quantitative terms. In poultry industry, these terms include for

example lost revenues; costs of vaccination, prevention, eradication, decontamination and restocking. These have been referred to as negative inputs (Thrusfield, 1995).

In Nigeria coccidiosis is one of the most important causes of mortalities in all farms. It inflicts the birds in both clinical and subclinical forms. The clinical form of the disease manifests through prominent signs of mortality, morbidity, diarrhea or bloody feaces and subclinical coccidiosis manifests mainly by poor weight gain and reduced efficiency of feed conversion and give rise to highest proportion of the total economic losses (Williams, 1999). Although coccidiosis is probably the most frequently reported disease of chickens worldwide (Biggs, 1982), there are considerable difficulties in arriving at a reliable figure for specific financial losses. There are very few reports on economic losses due to coccidiosis (Oyekole, 1984; Brauninus, 1987; Graat *et al*., 1996). The increase crowding of birds under mass production methods creates a favourable condition for the occurrence of coccidiosis. It is one of the top five poultry disease most frequently diagnosed in the field and laboratory representing 5-15% of all mortalities, with subclinical coccidiosis more common than clinical coccidiosis. Losses due to this form of the disease are heavy and cannot be estimated (Gordon and Jordan, 1982).

A mild coccidiosis infection kept under control is not very harmful and is actually necessary for creating immunity in replacement flocks and free ranging birds. However, a severe attack of the disease can cause weight loss, reduced egg production, morbidity and mortality (Lillehoj and Trout, 1996). Poultry coccidiosis caused by *E. acervulina, E. necatrix, E. maxima* and *E. tenella* are endemic in all parts of the country and affects mainly young growing birds (Lawal *et al*., 2008). In Nigeria, coccidiosis is causing

significant poultry losses. Coccidiosis was identified to be a major cause of both direct and indirect losses in all farms. Losses occurred in the form of mortalities, coccidiostat costs, reduced weight gains, reduced market value of affected birds, delayed off take and reduced egg production in layers (Gyorke *et al*., 2013). The disease also contributed to culling. Quantification of economic losses resulting from coccidiosis in small scale and large-scale poultry farms (Safari *et al.,* 2004). The total cost of coccidiosis in chickens in the United Kingdom in 1995 was estimated to be at least 55 US$ million of which 98.1% involved broilers (80.6% due to effects on mortality, weight gain and feed conversion, and 17.5% due to the cost of chemoprophylaxis and therapy). The costs of poor performance due to coccidiosis and its chemical control totaled 4.54% of the gross revenue from UK sales of live broilers (Williams, 1999).

2.11 Pathogenesis of *Eimeria tenella*

Eimeria tenella causes a fatal disease in chickens called caecal coccidiosis. It is caused due to extensive destruction of the caecal epithelium, with sloughing off the all and severe haemorrhage. The symptoms include bloody droppings, pale feces, and shanks, bloody vent and enlargement of caeca or with yellowish grey cheese-like ceres. In advanced stages, the infected bird shows restlessness, dropping wings, ruffled feathers, and unsteady gait (Conway and Mckenzie, 2007). Caecal coccidiosis under field conditions mostly in young chickens than in older ones.The range of age of susceptibility is from two weeks .to 15 months. Caecal coccidiosis outbreaks occur at the age of six to eight weeks (Conway and Mckenzie, 2007).

Edgar (1992) in a study on experimental infection reported that the heaviest mortality and greatest decrease in erythrocytes occurred in chicks one month old, heavy mortality also occurred in chicks aged two weeks and two months.In general,chicks rigidly isolated from infection remain fully and uniformly susceptible throughout their lives (Long *et al*., 1980). Under field conditions almost all chickens get early exposure to at least light infection and so nearly all chicks more than a few days old have some degree of resistance (Graat *et al*., 1996).

Edgar (1992) demonstrated that the presence of food in the digestive tract of birds at the time of infection reduced the severity of the disease. The number of oocysts resulting from an infection is not a true indication of the magnitude of infection. During the course of an infection, there are several factors which may cause a reduction in this potential including loss of merozoites, over crowding, and tissue damage which results in a loss of suitable cells.

Biggs (1982) showed that for each oocyst of *Eimeria tenella* administered in light infection approximately 10,000 oocysts are produced, and there is no direct correlation between the size of infective dose and the final degree of inition.Joyner and Norton (1983) reported that the severity of caecal coccidiosis depends on the number of sporulated oocysts that the bird receives.The prepatent period in *Eimeria tenella* infection is 7 days but the patent period varies viith individual infections.Under natural conditions, birds are normally infected repeatedly and therfore may pass oocysts for long periods of time.

The disease symptoms in caecal coccidiosis are closely related to the course of infection and, in general the degree of pathogenicity is related to the depth to which the cecal vrall is

parasitized. *Eimeria tenella* penetrates deeply and is very destructive (Conway and Mackenzie, 2007). During the development of the parasite, there is a migration of parasitized cells into the subepithelial region where they increase enormously in size. Much tissues are destroyed and sloughing of mucosa occurs at time of maturation of second generation schizonts as early as ninety six hours after infection and profuse hemorrhage occurs due to mechanical damage to the blood vessels. This bleeding is the most important effect of the parasitism (Tierney *et al*., 2007).

For experimental infections of coccidiosis, knovm numbers of sporulated oocysts are administered orally. Johnson and Reid (l970) induced caecal coccidiosis by inoculating *Eimeria tenella* oocysts subcutaneously, intravenously, intraperitoneally or intramuscularly in birds.

Mortality is likely to be heavy when profuse and continuous bleeding occurs for seven days post infection. The damage may also result from secondary bacterial infection of the caeca in which the epithelium is completely destroyed (Biggs, 1982). Hemorrhage is a great stress on the infected chicken and feeding and movement are at a minimum during this period. In a typical severe infection, bloody droppings will occur ninety six hours post infection and passage of large quantities of blood in the droppings on the fifth and sixth day post infection.Caecal coccidiosis is at its peak on day seven post infection.Chickens surviving nine days followjing infection will usually recover (Maxey and Page, 1977). A chronic condition may occur as the result of retention of a core of necrotic tissue in the caeca with consequent caecal dysfunction (Allam, 1989).

When chickens are raised on deep litter as in most parts of the world, the oocysts are not necessarily destroyed by the heat of fermentation, but due to the unfavorable environment are predominantly unsporulated. Appearance of fresh blood in the droppings and sudden death are of diagnostic value in caecal coccidiosis. Clotting of blood is prevented during the acute stages by some unknovm factor(s) and deficiency of vitamin K increases pathogenicity to *E. tenella* (Bafundo and Donovan, 1988). At necropsy, the bloody caeca and presence of developmental stages of *Eimeria tenella* confirm caecal coccidiosis. Oocysts presence is not indicative of disease because in *Eimeria tenella* infections, oocysts are rarely seen in an acute infection (Davis, 1973).

2.12 Clinical Signs of Chicken Coccidiosis

Clinical signs of coccidiosis are due to destruction of the intestinal epithelium and frequently, the underlying connective tissue of the mucosa. This may be accompanied by hemorrhage into the lumen of the intestine. Serum protein and electrolyte levels may be appreciably altered (Kahn *et al.,* 2008). Signs may include discharge of blood, dehydration, decreased growth rate to a high percentage of visibly sick birds, severe diarrhoea and high mortality. Feed and water consumption are decrease, weight loss, development of culls and increased mortality may accompany outbreaks (Biggs, 1982). The effects of coccidiosis are due to a number of factors, all of the observed effects are related to disruption of the epithelial cells lining the intestine by the release of developmental stages of the parasite. The main effects that cause economic losses are a decreased weight gain due in part to the malabsorption of nutrients through the gut wall. This effect causes an increased feed conversion ratio, which is the amount of feed

converted into body weight, because feed that is consumed is used inefficiently. Chickens that are infected with high levels of *Eimeria* species display symptoms such as droopiness and emaciation and may never achieve weight gain equal to their uninfected counterparts (Graat *et al.,* 1996).

Coccidiosis is generally acute in onset and is characterized by depression, ruffled plumage, and diarrhea. Birds infected with *E. tenella* show pallor of the comb and wattles and blood-stained caecal droppings (Simon, 2005). Several factors influence the severity of infection, some of these include; the number of oocysts ingested, nutritional status and age. Generally an increase in the number of oocysts ingested is accompanied by an increase in the severity of the disease. Different strains of a species may vary in pathogenicity, environmental factors affecting the survival of the oocysts, site of development within the host. Coccidia that develop superficially are less pathogenic than those that develop deeper. Young birds are generally more susceptible than older ones. Coccidiosis in chickens is generally classified as either intestinal or caecal. Most serious cases of intestinal coccidiosis are caused by *E. necatrix* and *E. acervulina*. Caecal coccidiosis is due to *E. tenella.* Coccidiosis occurs most frequently in young birds, older birds are generally immune as a result of prior infection. Severe damage to the caeca and small intestine accompany the development of Coccidia. Broilers and layers are more commonly infected, but broiler breeders, turkey and pheasant are also affected. Coccidiosis generally occurs more frequently during warmer (May to September) than colder months (October to April) of the year.

2.13 Pathogenicity of *Eimeria tenella*

Coccidiosis often lead to disturbances such as lowering nutrients absorption which in turn leads to disturbances in the ions and osmotic balance of the gut epithelium (Fitz-Coy and Edgar, 1992). Oocysts infect the cells of intestinal lining, replicate and cause them to burst thereby causing change in the gut morphology, reducing gut length and truncating the intestinal villi (Joyner and Norton, 1983). Ingested small number of oocyst lead to a subclinical infection with no obvious diagnostic clinical signs and subsequent immunity to re-infection. Pathology is largely associated with destruction of the epithelial lining of the infected part of the intestine which results in reduced ability for the digestion and absorption of nutrient by the bird (Long *et al.*, 1980).

The mucus gel layer overlying the gastrointestinal epithelium plays an important role in host–pathogen interactions. The initial interaction between *E. tenella* and host cells of the intestinal epithelium occur across this mucus interface. The relationship between *E. tenella* and avian mucin was examined (Tierney *et al*., 2004), in particular, the effect of purified intestinal regional mucin on parasite adherence and invasion *in vitro*. Secreted mucin from the chicken duodenum and caeca was purified by density gradient centrifugation and gel chromatography. *Eimeria tenella* adherence to chicken duodenal mucin was detected, whereas adherence to caecal or bovine mucin was not shown. Parasite invasion into epithelial cells was not influenced by bovine mucin, whereas chicken mucin purified from the duodenum and caeca significantly inhibited invasion. Inhibition of *E. tenella* invasion into cells by mucin from the duodenum was marginally greater than that of the caeca, but this was not significant (Tierney *et al*., 2004). The species important in broiler production include *E. tenella*, *E. maxima*, *E. acervulina*, and *E. mivati*, the species

important in breeder and layers are *E. burnetti* and *E. necatrix*. Seven species infect turkeys, the big three of concern are *E. meleagrimitis, E. adenoeides* and *E. gallapovonis* (Julie, 1999). *Eimeria tenella* is the well-known cause of caecal or bloody coccidiosis. It invades the two caeca and in severe cases may also parasitize the intestine above and below the caecal junction.Lesions of *E. tenella* are indicated as +1, +2, +3 and +4 scores (Conway and Mckenzie, 2007).

E. tenella +1 (Plate I), few scattered petechiae, which are reddish or purple in colour, are seen on the unopened caeca. There is no thickening of the caecal wall, the caecal contents usually show a normal brownish colour, although a slight amount of blood may be present, mild clinical signs may show in infected chickens (Conway and Mckenzie, 2007).

Eimeria tenella +2 is shown in Plate I, Petechiae, which are apparent on the serosal surface, are some what more numerous, bleeding, which appears on the fifth to seventh day of infection, is more marked on the mucosal surface than in a typical +1 score. In this example, bleeding is slightly more severe than in the usual +2. Except for the presence of some blood, the caecal contents are normal. Another more reliable characteristic in judging severity is the amount of thickening of the caecal wall, which is slight in this case. Clinical signs are apparent in infected chickens with these degrees of infection (Conway and Mckenzie, 2007).

E. tenella +3 is shown in Plate II. Bleeding is more severe, with clotting appearing in the distal end of the pouch. The clot becomes hardened as the sloughed mucosal surface joins the bloody material to form a core. There is an absence of normal caecal contents since the caeca become practically non functional. Marked thickening of the caecal wall occurs. The

serosa of the unopened caeca shows the petechiae as coalesced and eroding the entire surface. Huddling, chilling and bloody droppings constitute clinical signs (Conway and Mckenzie, 2007.

E. tenella +4 is shown in Plate II. There is severe bleeding, a much thickened caecal wall and erosion of the mucosal surface show up on the fifth day of infection. The unopened caeca is distended, with blood at the distal end, but is contracted and shortened. Chickens huddle and sometimes let out a high-pitched call, chickens cease feeding and drinking, death may come suddenly beginning on the fifth day, reaching peak on the sixth and extending through the seventh to the tenth day of infection. By the sixth to eighth day, the caecal core is hardened and may persist for another week or more. The core may take on a more whitish cast with a huge accumulation of sloughed mucosal surface material. Microscopic examination of scrapings would show many oocysts. Purple areas denoting the presence of gangrene and rupture of the caecal wall may occasionally occur at this stage, dead birds are scored +4 (Conway and Mckenzie, 2007).

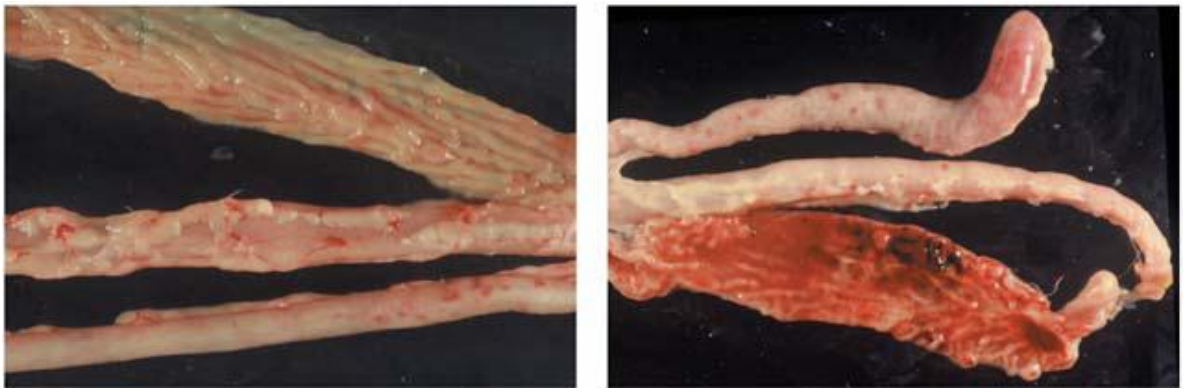


Plate I: *Eimeria tenella* infection scored + 1(left) and + 2 (right). Source: Conway and Mckenzie, 2007

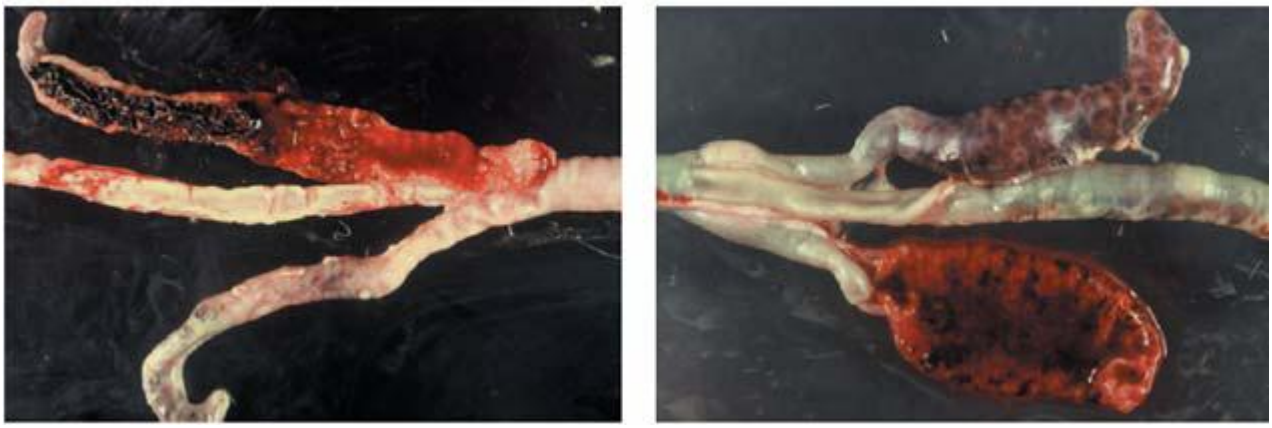


Plate II: *Eimeria tenella* infection scored + 3 (left) and + 4 (right). Source**:** Conway and Mckenzie, 2007.

2.14 Host Specificity of *Eimeria* Infection

Eimeria are highly host-specific. Rarely does one species of *Eimeria* complete an infectious cycle in more than one host species. However, exceptions have been noted under experimental conditions. For example, McLoughlin (1969) demonstrated that *E. meleagrimitis*, which normally infects turkeys, was able to infect chickens immunosuppressed by daily injections of dexamethasone. Similar treatment of turkeys, however, failed to make them susceptible to *E. tenella*, a chicken parasite. The underlying mechanisms of host specificity are not well understood, but most likely include genetic (Mayberry *et al*., 1982; Mathias and McDougald, 1987) nutritional/biochemical (Fry *et al*., 1984; Smith and Lee, 1986) and immune factors.

2.15 Diagnosis of Coccidiosis

2.15.1 Postmortem-clinical exmination

Eimeria tenella is the best known of poultry Coccidia, because of the easily recognizable lesions and often-spectacular losses it causes in commercial broilers or layer pullets. This species inhabits the caeca, causing a severe disease characterized by bleeding, high morbidity and mortality, weight loss, emaciation, loss of skin pigmentation, and other signs. Diagnosis is dependent upon finding caecal lesions with prominent blood and often-firm bloody cores and accompanying clusters of large schizonts and oocysts (Saif *et al*., 2003).

2.15.2 Faecal examination

Identification of *Eimeria* species oocysts in faeces is an easy and cheap way to diagnose many *Eimeria* species infections and to get an impression of the infection level, direct

smear method and both qualitative and quantitative techniques can be performed on faecal sample (Talebi and Mulcahy, 1995).

(i) Direct smear method

Identification of Coccidia oocysts is possible by using a direct smear method, where a thin smear of emulsified faeces is examined under a microscope. Direct microscopic examination of intestinal mucosa can only be used in animals, which have been culled or found dead. It can be used to find the intracellular and extracellular stages of Coccidia and other protozoa.

(ii) Faecal examinations by qualitative techniques.

There are different procedures for demonstrating coccidian oocysts in poultry faeces. The most widely used principle for concentration of parasite oocysts is flotation. Coccidia oocysts have a specific gravity, which is lower than that of plant residues in the faeces, the oocysts may be separated from other faecal particles by mixing the faeces with a fluid (saturated NaCl + glucose) in which the oocysts float, these procedures include test tube flotation and simple flotation.

(iii) Quantitative method for faecal examinations

The qualitative flotation techniques are used for nematode eggs, cestode eggs and Coccidia oocysts, have been elaborated to become quantitative, when the eggs are allowed to float in a special counting chamber, called the McMaster chamber. Many modifications exist and a Simple McMaster Technique and slightly more elaborated Concentration McMaster (Anders and Jorgen, 1998).

2.15.3 Haemagglutination inhibition assays

Intracellular *Eimeria* sporozoites are observed in epithelial cells which are attached on the cell surface of the epithelial cells .The carbohydrates present on sporozoites and lectin-binding sites on the surface of sporozoites were detected by means of peroxidase-conjugated lectins. By investigated parasite lectins of *E. tenella*, *E. acervulina* and *E. maxima* using hemagglutination inhibition assays, different stages of these parasites specific surface sugar lectins were found. The lectins found on the surface of the sporozoites play a role in determining the site of infection within the intestine of the host (Yoa *et al.*, 2003).

2.15.4 Enzyme linked immuno-sorbent assay

The enzyme-linked immunosorbent assay (ELISA) was adapted to detect antibodies to *Eimeria* spp. It is simple test and a reliable method for the determination of the exposure status of chickens to *Eimeria* species and detecting specific IgG and IgM antibodies in serum samples. The indirect enzyme-linked immunosorbent assay may be possible to discriminate between chickens actually infected with *Eimeria* species as indicated by high levels of antiparasite IgM, chickens which have been repeatedly exposed to *Eimeria* (as indicated by high levels of antiparasite IgG and unexposed birds.

The applicability of this ELISA, using sporozoite antigen of *E. tenella* to practical situations was substantially confirmed by Smith and Hayday (1998). Enzyme-linked immunosorbent assay analysis of serum pools having varying protective capacities revealed good correlations between passive protection and levels of anti-unsporulated oocyst, antisporulated oocyst, anti-merozoite and anti-gametocyte antibodies. Furthermore,

the cross-reactivity of these antigens with sera from birds infected with chicken *Eimeria* species is similar. The merozoite antigen is selected for further evaluation because it was easier to prepare. Discrimination between sera from birds experimentally infected with *E. tenella* and birds maintained in an *Eimeria*-free isolation facility was excellent. The enzyme-linked immunosorbent assay prove useful in monitoring infectivity in vaccination programs in layer and breeder flocks and for assessing the effectiveness of bio-safety measures in broiler flocks (Gilbert *et al*., 1998; Constantinoiu *et al.,* 2007)

2.15.5 Polymerase chain reaction

A polymerase chain reaction (PCR) assay, based on the amplification of internal transcribed spacer regions of ribosomal DNA, was developed for the chicken coccidian species-specific primers for the detection and discrimination of all *Eimeria* spp. that infect the domestic fowl is now available. The PCR assay provided a faster more simplified read-out compared to staining of amplified bands in an agarose gel with ethidium bromide (Beate et *al.,* 1999). The unsporulated oocysts of *Eimeria* species are difficult to differentiate. For identification of *Eimeria* species and variations in genomic DNA two primers corresponding to highly conserved regions of the 18S ribosomal DNA of the coccidian forward primer (BSEF) and the reverse primer (BSER) were chosen.

The internal transcribed spacer 1 (ITS-1) from within recombinant DNA (rDNA) genes was investigated to differentiate chicken intestinal coccidian to the genus and species level. The spacer separates the 3 end of the 18S ribosomal RNA gene from the 5 end of the 5.8S rRNA gene within individual rDNA transcription units. The five chicken *Eimeria* genus species-specific primers was designed as markers to identify species and sequences from

the vaccines coccivac-B and coccivac-D and from a Taiwanese strain of *E. tenella* . The cross-reaction would occur with the DNA from chicken intestinal contents and muscles, and the detection limit of PCR tested with pureline oocysts of *E. tenella* (Yao *et al.,* 2003).

2.15.6 A real-time diagnosis system

Using digital images of oocysts, an important goal in image analysis is to recognize and classify objects of interest in digital images. Objects can be characterized in several ways, e.g. by identifying their colors, textures, shapes, movements and position within images. Coccimorph, a real time system accessible through a web interface, (available at http://puma.icb.usp.br/coccimorph), allows the user to upload an image, detect the contour interactively and obtain a real-time classification. The framework of this system is divided into the following levels:

(i) Database: This level stores the feature vectors that compose the data set. Micrographs and isolated images are also stored and can be visualized through a webinterface.

(ii) Application: This is the developmental level of the system, which is divided into three modules: import subsystem, analysis subsystem, application and web server.

(iii) Client: This level is oriented to interact with the end-user, allowing for the visualization and uploading of images for diagnostic purposes. The analysis subsystem represents the kernel of the system and is responsible for the image pre-processing, feature extraction and pattern classification. This module was entirely developed, resulting in a rapid response of the system during the imageprocessing step, thus permitting a real-time processing through the web (Cesar *et al.,* 2007).

2.16 Control of Avian Coccidiosis

Anticoccidial feed additives have been used for more than 50 years to prevent or treat coccidiosis in poultry (Jeffers, 1997, Chapman, 1997 and Allen and Fetterer, 2002) and are classified anticoccidials as follows:-

2.16.1 Anticoccidial drugs

These are synthesized drugs, which include variant groups of completely different chemical classes:

1. Amprolium is good against *E. tenella* but is not very effective against *E.acervulina* and *E. maxima.*
2. Nicarbazin is a broad-spectrum anticoccidial, it is used in colder seasons or climatic areas and the drugs should not be used in birds older than 20 days because the possibility of strong growth depression. - Robendine is safe broad-spectrum anticoccidial but it must be used with caution because of it's potential fast resistance build up.
3. Halofuginone and Lerbek effects on *E. tenella* are coccidiostatic activity and no coccidiocidal effect, but good for control of *E. acevurlina*.
4. Clinacox (Dicluzuril). This has a broad-spectrum activity against all *Eimeria* species. The potential of *Eimeria* species, especially *Eimeria tenella* and *Eimeria maxima* to develop resstance to the drug is low. It is also used for “clean-up” program after the use of ionophore (Leu, 1999).

2.16.2 Sulfonamide products

Sulfaquinoxaline was introduced commercially as a poultry coccidiostat in 1948. It was not the first sulfonamide found active against *Eimeria* spp. in poultry, but its practical success in coccidiosis control firmly established the routine incorporation of anticoccidial drugs in poultry feed. The drug exerted a major impact on the worldwide production of poultry meat (Williams, 2008). Veterinarians and Animal scientist regularly use sulfonamides for therapeutic and prophylactic. Sulfadimethoxine, and sulfaquinoxaline are mainly used for prevention or treatment of poultry coccidiosis, and are generally co-administered in feed. The treatment of hens with sulfonamides-supplemented feed may result in sulfonamides residues being present in market eggs if these drugs have been improperly administered or if the withdrawal time for the treated hens has not been observed. To assure the food safety for consumers, the European Union has set a maximum residue limit for sulfonamides in foods of animal origin such as meat, milk, and eggs (Brussels, 1991). Improper use of these veterinary drugs in laying hens is of great concerns because the drug residues are turning up in eggs, which is an indispensable food for the consumers because it is highly nutritious, cheap and readily available.

A rigid residue monitoring of sulfonamides in eggs is therefore an important specific activity to guarantee the food safety. Discharging the waste of organic solvents is also a severe problem on the world scale. From the view point of the effect of organic solvents to environments and analysts, analytical methods for the monitoring should avoid the use of organic solvents (Guo-Zhen *et al,,* 2006; Ming-Ming *et al.,* 2008).The feeding of 2,500 parts per million (ppm) sulfaquinoxaline causes a severe anemia in chickens with hemorrhages on the legs, breast muscle, and in abdominal organs (Kahn *et al.,* 2008).

Toxicity is more likely to be observed when medication is given in the water during hot weather. Feeding 300-ppm sulfaquinoxaline to growing chickens for 8 weeks reduced the weight gain of female birds but adverse, effects were not observed when sulfaquinoxaline was administered to growing chickens at 300-ppm in various feeding schedules. Continuous feeding of 125-ppm sulfaquinoxaline was highly efficacious in preventing naturally acquired caecal and intestinal coccidiosis. The overall efficacy benefits of sulfaquinoxaline in comparison with othersulfonamides were attributed to the fact that it is more readily absorbed than other sulfonamides when given in the feed.

2.26.3 Ionophore products

Ionophores are the major group of poultry feed additives the polyether antibiotics commonly called Ionophores, six compounds have become available (Monensin, Laslocid , Salinomycin , Narasin , Maduramycin and Semduramycin), the mechanism of action of all ionophores is very similar since they mediate the transport of mono and divalent cations throw the membrane of the parasite, resulting in disturbance of its osmotic balance. Ionphores can be divided into three groups according to the precise of action and chemical structure; monovalent (Monensin, Narasin and Salinomycin), monovalent glycoside (Maduramycin and Semduramycin) and divalent (Laslocid). Laslocid and Maduramycin are more effective against *E. tenella* than Monensin, Narasin and Salinomycin (Leu,1999).

2.16.4 Polyether ionophores

They are produced by fermentation of *Streptomyces* or *Actinomadura* and they are the most commonly used agents, such as salinomycin, monensin, lasalocid and narasin. They act

through a general mechanism of altering ion transport and disrupting osmotic balance in the parasite.

2.16.5 Mode of action of anticoccidial drugs

Anticoccidials often have more than one biochemical effect, but each class of chemical compound is unique in the type of action exerted on the parasite and its development stage. Diverse modes of action have been described and this can be divided into several broad categories, according to Chapman (1997) and McDougald (1982, 2003).

(a) Drugs that affect cofactor synthesis

Several drugs affect biochemical pathways that are dependent upon an important cofactor. For instance, amprolium competitively inhibits the uptake of thiamine by the parasite.

(b) Drugs that affect mitochondrial function**:**

These drugs inhibit energy metabolism in the cytochrome system of the *Eimeria*. For instance, quinolones and clopidol inhibit electron transport in the parasite mitochondrion, but by different pathways.

(c) Drugs that affect membrane function**:**

Ionophores in common have the ability to form lipophylic complexes with alkaline metal cations (Na^{+}, K^{+}, and Ca^{++}) and transport these cations through the cell membrane and then affect a range of processes that depend upon ion transport, such as influx of sodium ions thus, causing severe osmotic damage. These drugs act against the extracellular stages of the life cycle of the *Eimeria*.

2.17 Resistance to Anticoocidial Drugs

In 1963, the World Health Organization (WHO) defined resistance as "ability of a parasite strain to multiply or to survive in the presence of concentrations of a drug that normally destroy parasites of the same species or prevents their multiplication". Resistance may be relative (increasing doses of the drug being tolerated by the host) or complete (maximum doses being tolerated by the host) (Chapman, 1982). Anticoccidial drugs added to the feed are a good preventive measure and are well adapted to large-scale use, but prolonged use of these drugs leads inevitably to the emergence of *Eimeria* strains that are resistant to all anticoccidial drugs, including ionophores (Chapman, 1994, 1997, 1998; Allen and Fetterer, 2002). Resistance can develop quickly, as in the case of quinolones and clopidol, or it may take several years for the Coccidia to become tolerant, as in the case of polyether ionophores (Chapman, 1997; McDougald, 2003).

2.17.1 Origin of resistance anticoccidial drugs

There are three key factors contributing to drug resistance in commercial poultry production (Jeffers, 1989; Chapman, 1997; Jeurissen and Veldman, 2002):

(a). The intense and the continuous use of anticoccidial drugs in the poultry industry providing the basis for changing gene frequency through genetic selection.

(b). Coccidia is ubiquitous in poultry facilities and the large reproductive potential forms a large reservoir of genetic variation, which leads to the development of drug resistance.

(c). The life cycle of *Eimeria* is complex and involves a period of asexual and sexual stages. The nuclei of the asexual stage of *Eimeria* contains haploid complement chromosomes. Most drugs are active against this haploid stage, resulting in the

removal of the most sensitive ones. This enables the more resistant ones to increase and thus rapidly becoming the dominant phenotype that spreads through the parasite population.

2.18 Poultry House Management

The high standard of flock hygiene, sanitation and poultry farm management helps in achieving optimal benefit from the use of anticoccidial drugs in preventing coccidiosis (Chapman, 1997). However, the sanitary practice alone is inadequate for complete elimination of coccidial oocysts. This is because of the following:

a) there have been too many failures in sanitary programs

(b) oocysts are extremely resistant to common disinfectants

(c) house sterilization is never complete

(d) an oocyst-sterile environment for floor-maintained birds could prevent early establishment of immunity and thus allow late outbreaks (McDougald, 2003).

2.19 Alternative for Anticoccidial Drugs

The constant and extensive use of the anticoccidial drugs for prevention and control of coccidiosis in poultry has been a major factor in the success of the industry. This beneficial use of anticoccidial drugs is attributed with a widespread drug resistance of Coccidia in the United States, South America and Europe (Jeffers, 1974 a & b; Litjens, 1986; McDougald *et al*., 1986; 1987; McDougald, 2003). The first line of defence against development of resistance is the use of shuttle or dual programs (two or more drugs employed within a single flock) and frequent rotation of drugs (rotation of different compounds between flocks) (Chapman, 1997; McDougald, 2003). The pressure by the consumers to avoid

chemotherapeutics, the high development costs and low profits have not encouraged the pharmaceutical industry to develop new anticoccidial products (Chapman, 1997). Thus, alternatives have been sought and are still being sought.

2.19.1 Early vaccines trials against *Eimeria* species

(a) Probiotics

Metchnikoff (1908 as cited by Hussain, 2010) proposed that the consumption of live microorganisms (mainly lactic acid bacteria) could improve intestinal health and well-being of the host. Probiotics was also defined as "a live microbial feed supplements which beneficially affect the host animal by improving its intestinal microbial balance" (Fuller, 1989). Probiotic preparations may consist of a single strain *Lactobacilli* or *Streptococci* or may contain any number up to eight strains (Fuller, 1989; Timmerman *et al.,* 2004). The use of probiotics aims to fasten the development of a stable and beneficial intestinal microflora, which will lead to improvement of intestinal health and modulate the immune system, enhancing host resistance to enteric pathogens (Jin *et al.,* 1996; 1998; 2000; Abdulrahim *et al.,* 1999; Zulkifli *et al.,* 2000).

Tortuero (1973) demonstrated the antagonism between Lactobacilli and enterobacteria and showed that lactobacilli reduced the severity of clinical signs in *E. tenella* infection. Dalloul and Lillehoj (2005) reported that a *Lactobacillus* containing diet fed to broilers infected with *E. acervulina* resulted in an immunoregulatory effect on the local immune system and improved the broilers' resistance to *E. acervulina* infection. Furthermore, it has been reported that *lactobacillus* species inhibit the invasion of *E. tenella in vitro* (Tierney *et al*., 2004). Recently, Lee *et al*. (2007) reported that *Pediococcus acidilactici* effectively

enhanced the resistance of birds and partially protected against the negative growth effects associated with coccidiosis.

(b) Prebiotics

Gibson and Roberfroid (1995) defined a prebiotic as "a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or limited number of bacteria in the colon that can improve the host health". Prebiotics have the advantage, when compared with probiotics, that they are targeting the bacteria already present and hence those that are adapted to the gastrointestinal tract environment. Many studies have proved that the non-digestible polysaccharides inulin, oligofructose, and oligomannose, enhance the growth of the beneficial bacteria (*Bifidobacteria and Lactobacillus)* and reduce that of the pathogenic bacteria (*E. coli* and *Salmonella*) and also stimulate the immune system of the host (Hikosoka *et al.,* 2007; Wang and Gibson, 1993; Gibson and Roberfroid, 1995; Gibson *et al.,* 1995; Gibson and Fuller, 2000; Cummings and MacFarlane, 2000).

Mannanoligosaccharides (MOS), derived from the cell wall of the yeast, can be considered as prebiotics. MOS is non-digestible and is utilized by lactic acid producing bacteria (Delzenne, 2003). MOS also competes with mannose-specific binding of type-1 *fimbriae* of pathogenic, gram-negative bacteria such as *E. coli* and *Salmonella*, resulting in a reduction of their colonization (Ofek *et al.,* 1977 and Fernandez *et al*. (2002) reported an increase in faecal *Bifidobacteria* and a reduction in susceptibility to *Salmonella enteritidis* colonization in young chickens fed a diet supplemented with MOS. Addition of MOS to the diet of broilers reduced the severity of the infection due to either *E. tenella* alone or a mixture of *E. acervulina, E. maxima* and *E. tenella* (Elmusharaf *et al*., 2006).

2.20 Vaccines for *Eimeria* Species

Vaccines are the most valuable public health tools that have been developed by man (Payette and Davis, 2001). The development of resistance of coccidia to anticoccidial drugs (Chapman, 1997; Williams, 2002), the concern about drug residues in poultry products (McEvoy, 2001; Young and Craig, 2001), the pressure imposed by consumers to avoid chemotherapeutics and the recent announcement by the EU to ban several anticoccidial drugs used in broilers (Farrant, 2001), have led to interest in the vaccination of poultry against coccidiosis. In addition to the vaccines currently available, many others are under development (Chapman *et al.*, 2005). Jeurissen and Veldman (2002) listed factors making coccidiosis as a disease that can be controlled by vaccines. These factors are:

1. immunity to avian coccidiosis is strongly species-specific.
2. coccidiosis infection induces a quick and strong protective immunity.
3. lack of antigenic variation in *Eimeria* species.

However, as described above, *Eimeria* exhibit a complex life cycle comprising stages both inside and outside of the host. During the in-host stage, there are both intracellular and extracellular stages and both asexual and sexual reproduction. This complexity provides the immune system with only three moments to inhibit *Eimeria* development. The first is when the sporozoites search for a site of penetration and actually bind with the epithelium. The second is when the sporozoites are in the villus epithelium, inside and between intraepithelial leucocytes. The third moment of possible attack by the immune system is during the passage of the lamina propria into the crypt epithelium (Jeurissen and Veldman, 2002).

There are four major brands of vaccines commercially available, and they are based on the use of wild type (Coccivac® D/B and Immucox®) and attenuated (Paracox® and livacox®) *Eimeria* species (Williams, 1998; 2002; Chapman *et al*., 2005). The non-attenuated vaccines contain a mixture of oocysts of wild-type-strain *Eimeria* that will not produce pathogenic effect, but induce immunity. The methods of administration of vaccines have been reviewed by Williams (2002). In the past, vaccines were applied via drinking water or feed when the chickens are about one-week of age, but recently the method of vaccination is a single dose at day one with Coccivac D® (Chapman and Cherry, 1997a), Immuncox® (Chapman and Cherry, 1997b) and Coccivac B® (Chapman *et al*., 2005). Administration of vaccines as a single dose at day-one of age is important in initiating immunity as early as possible in broilers as they are reared only to about 6 weeks of age. However, some studies indicated that vaccination on day one could not evoke a strong immunity since the immune system in young chicks is immature (Rose, 1987). In contrast, other studies have shown that chicks infected at day one of age indeed are capable of building an effective immunity (Lillehoj, 1988; Bafundo and Jeffers, 1990; Chapman and Cherry, 1997a). Many scientists have reported that even embryos have a functional immune system (Fredericksen *et al*., 1989; Doelling *et al*., 2001).

There are various methods of administration of coccidial vaccines, including intra-ocularly (Coccivac®), by hatchery spray (Coccivac® and Nobils®), by edible-gel (Immucox®), or by spraying on feed (Coccivac®, and Paracox®) (Chapman, 2000; Chapman *et al*., 2005 and Williams 2002). Immunological protection against *Eimeria* is strongly species specific (Rose, 1973; 1978), a number of species have been incorporated in vaccines, varying from

two species (*E. acervulina* and *E. tenella* as in LivacoxD®) to eight species (*E. acervulina, E. brunetti, E. maxima, E. mitis, E. mivati, E. necatrix, E. praecx,* and *E. tenella* as in CoccivacD®) (Williams, 1998). However, Williams (1998), and Chapman (2000) recommended the inclusion of *E. acervulina, E. maxima,* and *E. tenella* and the exclusion of *E. brunetti,* and *E. necatrix* in vaccines as the latter two species rarely infect younger chickens.

Following vaccination, immunity is initially stimulated by the vaccine oocysts and is subsequently boosted and maintained by multiple re-infections initiated by the viable oocysts in the litter either originating from the vaccine or from local wild-type strains (Chapman, 1997; Williams, 2002; Chapman *et al*., 2005). This synchrony of infection development is called "trickle" infection and has been shown to be crucial in stimulating solid protective immunity (Joyner and Norton, 1973; 1976; Nakai *et al*., 1992; Chapman and Cherry, 1997a).

2.20.1 Types of vaccines

Coccidial vaccines licensed in the US include Coccivac, Immucox and Advent vaccine. These vaccines can actually cause some lesions and occurrence of coccidiosis in birds because they are not attenuated or weakened in some way. It is a controlled occurrence, but it may be necessary to treat for secondary gut disease, using antibiotics or alternatives such as probiotics. Coccidiosis vaccines used in Europe are attenuated. They are altered because the coccidia used in the vaccine are designed to mature quickly and have a short life cycle and low fertility. They are not pathogenic disease causing and are more costly to produce

than the non attenuated vaccines. They include Paracox, Livacox, and Viracox, which are marketed in other countries but not currently in the US.

More types of vaccines are likely to be developed, because the government approval process is much cheaper for vaccines than for anticoccidial drugs. Anticoccidial vaccines include mixtures of species of *Eimeria* that affect chickens. It is especially important to include the three types that cause the most damage in chickens; *E. acervulina*, *E. maxima*, and *E. tenella* (Fanatico, 2006).

2.20.2 Methods of vaccine applications

Spray cabinets; these are used at hatcheries on day-old chicks, resulting in 90 to 95 percent of chicks exposed to the vaccine. Edible gel; gel pucks are placed in transport crates or on the floor of the house when the chicks arrive. Feed spray: vaccines are mixed with water in a garden pressure-sprayer and sprayed on a 24-hour supply of feed (Fanatico, 2006). The chicks should be slightly water-starved to encourage them to drink. Since oocysts are heavy and fall to the bottoms of drinkers, they are mixed with a suspension agent to keep them evenly distributed. This method can be used for older chicks. Vaccines cannot be given through proportioners or nipple drinkers. It is important to apply vaccines uniformly to ensure the birds get equal exposure. If birds receive too much of a non-attenuated vaccine, the parasites can cause lesions. If attenuated vaccines are not given in adequate doses, the birds will be susceptible to field strains of the coccidia. The environment must allow the oocysts to sporulate, since the goal of vaccination is to introduce the parasite in small numbers. Litter should be damp but not wet after vaccination; birds excrete fresh

oocysts onto the litter. Birds then eat these (second cycle) oocysts. Two cycles of replication are needed for good protection.

2.21 Dietary Modulation of Coccidia

The study of the interactions between diet composition and Coccidia is not a new area **of** interest. Before the availability of effective anticoccidial drugs, recommendations for coccidial control included the formulation of diets that were considered capable of reducing the severity of infection such as diets containing skimmed milk, buttermilk, or whey (as cited by Beach and Corl, 1925; Becker, 1937 as cited by Hussain, 2010). But due to the development of the efficient, low-cost anticoccidial drugs caused lesser interest in dietary modulation. However, with the appearance of resistance to coccidiostats, the consumers' concern, and the expected regulations to ban the coccidiostats in the future, the possible role of nutrition has recently attracted interest (Allen *et al*., 1998; Gabriel *et al*., 2006).

2.21.1 Vitamins and minerals

Several vitamins influence the immune status and the resistance of the host against *Eimeria* infections. Many studies reported that vitamin A deficiency depresses T-lymphocyte response to mitogens (Friedman and Sklan, 1989a) and reduces specific antibody production to protein antigens (Friedman and Sklan, 1989b). Recently, Dalloul *et al.* (2002) reported that vitamin A deficiency in chickens caused alteration in the IEL subpopulation, reduced the local cell-mediated immunity, and lowered the ability of birds to resist *E. acervulina* infection. Vitamin E and selenium generally improve resistance to

coccidiosis, improve weight gain (Colnago *et al.*, 1984a; El-Boushy, 1988), and reduce mortality due to *E. tenella* infection (Colnago *et al*., 1984b).

Vitamin C is known to possess immunity-enhancing effects in chickens and positive effect on birds' performance during coccidial challenge has been observed (Attia *et al*., 1979), but it had no effect on the lesion scores due to *E. tenella* or *E. acervulina* infection (Webber and Frigg, 1991). Additional, Waldenstedt *et al*. (2000b) found that feeding a diet with extra vitamins A, C, D_3, K, and selenium had no beneficial effects on the performance of chickens with subclinical infection caused by *E. maxima,* and *E. tenella*. Additional, the authors reported that performance in the birds supplemented with vitamins was even poorer than in birds fed the control diet. These results are in contrast with previous work of Colnago *et al*. (1984b) who fed 0.025 or 0.50 mg Se/kg of diet, noted a reduced mortality, an increase in body weight, and improved immunity against *E. tenella*.

2.21.2 Products rich in n-3 fatty acid

The n-3 fatty acids are polyunsaturated fatty acids, the major fatty acids being eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), found abundantly in fish oil, and alpha-linolenic acid (ALA), being a major component of flaxseed oil. As sighted by Allen *et al.* (1996 a & b; 1997a), they reported that fish-liver oil exerts favourable control on the course of coccidiosis. They also performed a series of experiments using fish oil, flaxseed oil and flaxseed in diets fed to male chickens from day 1 of age through 3 weeks of age and challenged with *E. tenella* at 2 weeks of age. The authors reported a significant reduction in caecal lesion scores and in the histological examination, a

significant reduction in the degree of parasitaization and retarded development of the *E. tenella* parasite was observed. The suggested mode of action is that the n-3 fatty acids infiltrate the tissues of the parasite, which in turn become more susceptible to oxidative attack by phagocytic cells.

Additionally, n-3 fatty acids have been shown to enhance the immune response in birds infected with *E. tenella*. However, little if any response was seen in the birds' performance, which is of most importance in poultry production. The n-3 fatty acids were proven ineffective against moderate or severe infection with *E. maxima*, and did not counteract reduced body-weight gain and lesion scores. The reason for the differences in response between these two *Eimeria* species to dietary n-3 fatty acids is not yet known (Allen *et al*., 1997a).

2.21.3 Betaine supplementation

Betaine supplementation has been shown to have positive effects on the water balance of broiler chicks stressed by high ambient temperature or coccidiosis (Augustine and Danforth, 1999), and to protect the cells from osmotic stress, allowing them to continue regular metabolic activities under conditions that would normally inactivate the cell (Ko *et al*., 1994). Augustine *et al.* (1997) reported that betaine, in combination with the ionophore and salinomycin had a significant positive effect on the performance of chickens infected with *E. acervulina*, *E. maxima*, and *E. tenella*, the effect being greater than that mediated by betaine or salinomycin alone. Moreover, the combination resulted in a slight decrease in development and invasion of the epithelium by *E. acervulina*, while there was an increase in the invasion of *E. tenella*.

However, the diet supplemented with betaine alone decreased the invasion of *E. acervulina* and *E. tenella* as indicated by the number of sporozoites present in the intestinal epithelium after the challenge. Klasing *et al.* (2002) later clarified this effect when they found that chickens fed betaine had more lymphocytes in the epithelium and in the lamina propria during *E. acervulina* infection than those fed the diet without betaine. This effect of betaine could result in more effective clearance of sporozoites that explain the decreased numbersin the epithelium as reported by Augustine *et al.* (1997), while Waldenstedt *et al.* (1999) found that betaine as a single feed supplement significantly improved chickens' body weight and tended to reduce the feed conversion ratio during coccidiosis infection. When betaine was used in the combination with the ionophore narasine, betaine showed no effects on birds' performance when *Eimeria tenella* was the major pathogenic species. The exact action of betaine is not fully understood. Augustine *et al.* (1997) suggested that betaine might increase performance in chickens infected by coccidiosis by inhibition of coccidial invasion and indirectly by supporting intestinal structure and function that could enhance the ability of the infected chickens to with stand coccidial infection.

2.21.4 Whole wheat

The use of whole grains in broiler feeds is a frquent practice in Europe (Forbes and Covasa, 1995). Many studies indicated that offering broilers a whole cereal grains and balanced pellets greatly reduced the severity of infection with *Eimeria* as judged based on the reduction in output of oocysts (Cumming, 1987; 1992; 1994). Waldenstedt (1998) and Banfield (1999; 2002) investigated the effects of whole wheat inclusion in broiler feeds with or without access to grit, and they observed no significant differences in faecal oocyst

yields, lesion scores, or performance in birds infected with *E. tenella* or *E. maxima*. They concluded that the decrease in output of oocysts as caused by inclusion of whole cereals in the diet, and observed in the previous experiments, was not due to the increase in the viscosity of the digesta or the crushing of oocysts by an active gizzard and that whole wheat addition to the diet of broiler chickens provides no control of coccidiosis.

2.21.5 Exogenous enzymes

The use of exogenous enzymes in food processing started as early as 1900 and the majority of the enzymes have been derived from fermentation by microorganisms (Clarkson *et al*., 2001). When broilers fed diet rich in wheat, barley, oat, or rye, the presence of non-starch polysaccharides (arabinoxylans and β-glucans) can give rise to high viscosity in the small intestine thereby decreasing the contact of endogenous digestive enzymes and its substrates. This results in a decrease in absorption and broilers' performance, and increase in the size of the GIT, pancreas, and the liver (Choct and Bedford, 1999; Bedford, 2000; Choct and Sinlae, 2000; Wang *et al.* 2005; Yim *et al*., 2011) reported an improvement in broilers' performance, a reduction in the size of digestive organs and the GIT size, and an increase in the total volatile fatty acids in the caecum, when a wheat-based diet was supplemented with the 200 mg exogenous enzymes xylanase or β-glucanase per kg feed. Addition of exogenous xylanase has been found to improve the performance and to reduce ileal digesta viscosity in *Eimeria*-infected birds (Cumming, 1992; 1994; Morgan and Bedford, 1995). It was concluded that intestinal viscosity and the size of the gizzard might affect the severity of the *Eimeria* infection. However, others did not observe effects of increased intestinal digesta viscosity on the severity of the *Eimeria* infection, when a large

increase in viscosity was being induced by the inclusion of carboxymethyl cellulose in the feed (Van der Klis *et al*., 1993; Banfield *et al*., 1999; 2002; Waldenstedt *et al.,* 2000a).

2.21.6 Electromagnetic fields

Electromagnetic fields (EMF) have been in use as therapeutic modalities for at least 40 years. It is well known that selected electromagnetic fields (EMF) can have beneficial effects on bones, joints, and neurological disorders, as well as wound healing (Cane *et al.,* 1993 and Montesinos *et al.,* 2000). Anti-inflammatory aspects of EMF exposure have been reported to be due to the activation of A_2A adenosine receptors in human neutrophils (Vallbona and Richard, 1999). Generally, inflammation is characterized by massive infiltration of T lymphocytes, neutrophils and macrophages into the damaged tissue (Gessi *et al*., 2000).

In earlier studies, it has been reported that EMF mediate positive effects on wound healing, controlling the proliferation of inflammatory lymphocytes, and therefore demonstrating beneficial effects on inflammatory disease (Jasti e*t al.,* 2001). Several authors (Blank *et al.,* 1992; Goodman *et al.*, 1994; Mevissen *et al.,* 1998) have discussed the effects initiated by various EMF signals and stated that EMF causes stress at the cellular level and that this leads to production of cytokines and consequently a biological response, including an immune response. Recently, Elmusharaf *et al.* (2006) reported that exposure of broiler chickens to EMF antagonized the effects of coccidial infection in birds infected with a mixture of sporulated oocysts containing *E. acervulina, E. maxima,* and *E. tenella*. It was found that the severity of the intestinal lesions mediated by *E. acervulina* and *E. maxima* were reduced in the EMF-treated birds.

2.22 Natural Additive and Herbs

A number of natural herbs have been tested as anticoccidial dietary additives. Artemisinin isolated from *Artemisia annua*, is a naturally occurring endoperoxide with antimalarial properties. It has been found effective in reducing oocyst output from both *E. acervulina* and *E. tenella* infections when fed at levels of 8.5 and 17 ppm in starter diets (Allen *et al.,* 1997b). The mode of action is thought to involve oxidative stress. Extracts from 15 Asian herbs were tested for anticoccidial activity against *E. tenella* and the test criteria were survival rate, bloody diarrhoea symptoms, lesion scores, oocyst output, and technical performance. Practical applications of these findings, such as the use of the products in starter rations or combinations of them with current anticoccidials or vaccines, appear possible and need to be investigated (Allen and Fetterer, 2002). Thus far, chemoprophylaxis and anticoccidial feed additives have controlled the disease but the situation has been complicated by the emergence of drug resistance (Abbas *et al*., 2008; Abbas *et al*., 2011a) and their potentially toxic effects on the animal health (Nogueira *et al*., 2009).

Furthermore, drug or antibiotic residues in poultry products may be potentially hazardous to consumers. Another approach for coccidiosis control is the vaccination of birds with live *Eimeria* oocysts, but, in cases of poor management, these vaccines can trigger severe reactions that may affect the performance of flocks, mainly in broilers because of their rearing period (Chapman, 2000). As a result of this drawback of live vaccines, attenuated vaccines (with reduced pathogenicity) have been developed, but these are expensive to produce.

2.22.1 Botanicals and coccidiosis

Cost effective alternative strategies are being sought for more effective and safer control of avian coccidiosis (Abbas *et al*., 2011b). The use of botanicals has played a major role in the control of avian coccidiosis, as they are not only natural products but may comprise new therapeutic molecules to which resistance has not yet developed. The use of botanicals as anticoccidial remedies, therefore, holds promise as an alternate in the control of coccidiosis.

2.22.1.1 Aloe species

Aloes are believed to have several medicinal properties and are used to treat various ailments. There are more than 360 known *Aloe* species, but the most recommended type of *Aloe* in controlling coccidiosis is *Aloe excelsa* (Gadzirayi *et al*., 2005). Gadzirayi *et al*. (2005) reported that the anticoccidial effects of *A. excelsa* were comparable with sulphachlopyrazine sodium monohydrate in terms of improved live weight gains and reduction in oocyst output in infected broiler chickens. Other species of *Aloe* plant such as *Aloe vera* have also been reported to have anticoccidial activities.

Aloe vera treatments show toxic effects on the intestinal tract by benefiting microflora and reducing bowel putrefaction as well as reducing inflammation (Bland, 1985). An *in vitro* trial was undertaken to determine the effect of three concentrations (15%, 30%, and 45%) of *A. vera* and *A. spicata* on the inhibition of the sporulation of avian coccidia oocysts (Marizvikuru *et al*., 2006). Both extracts showed a concentration-dependant anticoccidial effect, however, *A. spicata* inhibited sporulation to a greater extent than *A. vera.* In another

study (Yim *et al.*, 2011) dietary supplementation of *A. vera* resulted in significantly lower gut lesion scores and reduced faecal oocyst shedding of *E. maxima* in broiler chickens.These authors (Yim *et al*., 2011) suggested that reduced faecal oocyst shedding, a protective role against *Eimeria* infection, in *Aloe*-based chicken diets could be associated more with cell-mediated responses than antibody responses.

2.22.1.2 Artemisia species

Artemisia is a large, diverse genus of plants with between 200 to 400 species belonging to the daisy family Asteraceae. The most common species is *Artemisia annua* which has been reported for its antiparasitic activities. *A. annua* is a common type of wormwood botanical anticoccidials: Abbas *et al*. (2004) and Oh *et al*. (1995) conducted the first experimental trial to evaluate the anticoccidial activity of *A. annua* extracts against *E. tenella* in chickens. *A. annua* extracts showed the anticoccidial activity in terms of improved weight gain, improved feed conversion ratio and reduced lesion scores in infected chickens. Later, Allen *et al*. (1997a) reported a significant anticoccidial effect of *A. annua* against *E. tenella*, measured as reduced lesion scores, when fed to broiler chickens for three weeks as dried leaves at a dietary concentration of 5% (equivalent to 17 ppm pure artemisinin).

The pure form of artemisinin, fed for a period of 4 weeks at levels of 2, 8.5 and 17 ppm, significantly decreased oocyst output from single and dual species infection with *E. tenella* and *E. acervulina*. Moreover, artemisinin isolated from *A. sieberi* was also found to be effective against *E. tenella* and *E. acervulina* but not against *E. maxima* (Arab *et al*., 2006). So far, a limited amount of work has been carried out to determine the anticoccidial effect of *Artemisia* spp. in layer chickens. Brisibe *et al*. (2008) studied the effect of feeding 20% dried pulverized *A. annua* leaves against *E. tenella* both in broiler and layer chickens. The

anticoccidial effects of diets containing *A. annua* leaves were almost equal to the commercial anticoccidials both in broiler and layer chickens.The proposed mechanism of action of artemisinin involves cleavage of endoperoxide bridges by iron producing free radicals (hypervalent iron-oxo species, epoxides, aldehydes, and dicarbonyle compounds) which damage biological macromolecules causing oxidative stress in the cells of the parasite (Allen *et al*., 1998).

2.22.1.3. Azadirachta indica (neem) plant

Azadirachta indica (neem) plant is commonly available in Asian and African countries and is well known in the therapy of a number of infectious diseases including coccidiosis. Neem fruit, at a concentration of 150 g/50 kg feed, has been found to have anticoccidial effects against *E. tenella* infection by reducing oocyst excretion and mortality in broiler chickens (Tipu *et al.*, 2002). In addition to the anticoccidial effect of neem fruit, some reports have shown the anticoccidial activity of an aqueous extract of neem leaves against *E. tenella* alone (Takagi *et al.*, 2006) as well as in a mixed infection (Biu *et al*., 2006), which was comparable to the commercial anticoccidials amprolium and baycox.

The exact mechanism of action of neem against coccidian parasites is unknown, but a report by the National Research Council (1992) suggested that aqueous neem leaf extract, when taken orally, produces an increase in red cells, white blood cells and lymphocyte counts thus enhancing the cellular immune response, increasing antibody production and so most pathogens can be eliminated before they cause the symptoms associated with disease. Further research is needed to determine the maximum safe levels of neem

supplementation because the higher doses, due to its bitterness, may show adverse effects on feed intake which will influence the performance parameters of birds.

2.22.1.4 *Beta vulgaris*

The beneficial effects of incorporating sugar beet (*Beta vulgaris*) solids in animal feeds on livestock growth and overall performance have been known for a long time. One of the active ingredients is betaine which protects cells against osmotic stress by stabilizing cell membranes through the maintenance of osmotic pressure in the cells.

2.22.1.5 Curcuma longa

Curcuma longa L. (Zingiberaceae), commonly known as turmeric, is a medicinal plant widely used and cultivated in the tropical regions. In developing countries like Pakistan, poultry farmers provide turmeric powder as a feed additive for the control of coccidiosis in broilers (Abbas *et al*., 2010). The active compound of turmeric is the phenolic compound curcumin, which has been shown to have antioxidative, anti-inflammatory and immunomodulatory properties (Allen *et al.,* 1998). In an experimental study, the anticoccidial effect of dietary supplementation of 1% curcumin was observed in chickens after infection of *E. maxima* and *E. tenella* species. Improved weight gain, reduced lesion scores and oocyst counts were shown only against *E. maxima*. A significant reduction of plasma $NO2^{-}$ and $NO3^{-}$ was found only in *E. maxima*-infected and curcumin-treated birds, and hence provides a possible explanation for the difference in anticoccidial activity found for both *Eimeria species* (Allen *et al.,* 1998). Later, Abbas *et al*. (2010) reported that dietary supplementation with 3% *C. longa* powder was effective against a mild infection of *E. tenella*.

The proposed mechanism of action of *C. longa* (curcumin) involves the induction of oxidative stress against coccidia. Further studies are required to determine the possible anticoccidial activity of different concentrations of whole *C. longa* and its active ingredient curcumin against different *Eimeria species* in poultry.

2.22.1.6 Echinacea purpure

Echinacea and its different preparations contain a variety of active substances such as flavonoids, polysaccharides, glycoproteins, alkamides, cinnamic acids, essential oils and phenolic compounds (Liu *et al*., 2007; Zhai *et al*., 2007) which are effective in treatment of various ailments and are proven to be beneficial in promoting immunity (Bauer, 1999). This plant is known to have anti-inflammatory, antioxidant and immunomodulating properties that may be linked to its anticoccidial effects (Zhai *et al*., 2007). In an experimental study (Allen, 2003), ground root preparations of *E. purpurea* (0.1% -0.5%) were offered to broilers for two weeks which ameliorated weight gain reduction and birds had fewer coccidial lesions after a mixed challenge infection with *E. acervulina, E. maxima, E. tenella* and *E. necatrix*. The exact mechanism of action is still unknown, but because of its antioxidant properties *Echinacea* therapy may induce a state of oxidative stress against *Eimeria* species.

2.22.1.7 Origanum vulgare

The essential oils of *Origanum vulgare* are well known for their antiprotozoal activity (Milhau *et al*., 1997). Giannenas *et al*. (2003) carried out a study to examine the effect of dietary supplementation of *O. oregano* (*O. vulgare*) essential oil on performance of broiler

chickens experimentally infected with *E. tenella*. It was concluded that *O. oregano* essential oils, mainly carvacrol and thymol, had anticoccidial effects against *E. tenella*. Some studies suggest that vaccination against coccidiosis, in combination with *O. oregano* containing compounds, may be an alternative control method for intestinal health in chickens (Waldenstedt, 2000a). In addition, some studies suggest the use of dried oregano leaves as a natural herbal growth promoter for early maturing of birds (Bampidis *et al*., 2005). The dietary supplementation of *O. oregano* containing plants like *O. vulgare*, therefore, seems equally effective for maintaining the performance and reducing pathogenic parameters in infected birds.

2.22.1.8 Saccharum officinarum

Sugar cane (*Saccharum officinarum*) extract (SCE), a well known natural immunostimulant, is reported to have protective effects against *E. tenella* infection in chickens (El-Abasy *et al*., 2003). Some studies (Hikosaka *et al*., 2007) showed a significant increase in the number of IgM- and IgG plaque-forming cell responses of peripheral blood leukocytes (PBL), intestinal leukocytes, splenocytes, in addition to significantly higher phagocytic activity of PBL and antibody responses in chickens that had been orally administered with either sugar cane extract (SCE) or the polyphenol-rich fraction (PRF). Most recently, Awais *et al*. (2011) reported the immunotherapeutic effects of sugar cane extract against mixed *Eimeria* species in broiler chickens. The results of these studies suggested that sugar cane extract has an immunostimulating effect in chickens and their administration may augment protective immunity against coccidiosis.

2.22.1.9 Triticum aestivum

The *supplementation* of whole *Triticum aestivum* (wheat) grains in broiler feeds is common practice in Europe (Forbes and Covasa, 1995) because dietary fibre anti- oxidants may actually quench the soluble radicals that are continuously formed in the intestinal tract (Bao and Choct, 2010). Some reports (Allen *et al*., 1998) have noted the protective effects of whole cereal grains against coccidiosis in broiler chickens measured as a reduction of oocyst output. However, Waldenstedt *et al*. (1998) and Banfield *et al.* (2002) investigated the effects of whole wheat inclusion in broiler feeds with or without access to grit, and observed no significant differences in oocyst counts of mixed *Eimeria species*. They concluded that the reduction in output of oocysts by supplementation with whole cereals in the diet was not a result of the crushing of oocysts by an active gizzard or the increase in the viscosity of the digesta. Furthermore, they concluded that the whole wheat supplementation provided no control of coccidiosis in broiler chickens.

2.22.1.10 Yucca schidigera

Plant extracts with high saponin content are a good source of natural antimicrobial compounds. *Yucca schidigera* is a major source of natural saponins that cause the inhibition of protozoan development by interacting with the cholesterol present on the parasite cell membrane, thus resulting in parasite death (Wang *et al.*, 1998). Some studies have shown a beneficial and synergistic effect between the coccidiosis vaccine and the *Y. schidigera* extract in improving weight gains, feed conversion ratio and maintaining the integrity of the intestinal villi in chickens (Alfaro *et al.*, 2007). These improvements in the performance parameters of birds may be the result of the potential of saponins (extracted from the *Y. schidigera*) to improve the absorption of nutrients by the intestinal mucosal

surface (McAllister *et al*., 1998). These saponins are steroidal glycosides with strong surfactant activity, reducing the superficial tension of fluids and allowing better absorption of nutrients by the intestinal epithelium.

2.23 Treatment Programme for Coccidiosis Control

2.23.1 Shuttle or dual program

The use of one product in the starter and another in the grower feed is called a shuttle program in the US and a dual program in other countries. The shuttle program usually is intended to improve coccidiosis control. Intensive use of the polyether ionophore drugs for many years produced strains of coccidia in the field that have reduced sensitivity to the ionophores. It is a common practice to use another drug such as nicarbazin or halofuginone in the starter or grower feed to bolster the anticoccidial control and take some pressure off the ionophore. The use of shuttle programs is thought to reduce buildup of drug resistance. In 1988, approximately 80% of the US producers used some type of shuttle program (Saif *et al.*, 2003), in which two compounds usually a synthetic agent(such as Incarbazin) and Ionophore (such as Salinomycin) are employed successively in single flock. During 1999 in the US, shuttles involving synthetic drugs followed by Ionophores were employed by approximately 25% of broiler complexes (Chapman, 1999).

2.24 Future Hazards of Anticoccidial Residues in Broilers Meat Tissues to Man

Anticoccidial drugs play an important role in animal production, especially in intensive broiler production. They are used for disease prevention and therapy, as well as for their growth-stimulating effect. These drugs add to the recovery of animals from protozoal endoparasites, increase breeding productivity and decrease economic losses caused by

coccidiosis. However, mass and long-term administration of these substances has brought problems connected to the occurrence of unfavorable residues in animal products for human consumption.

The residues of anticoccidial drugs represent a potential risk to human health. Proper administration of these substances will ensure minimal content in animal products that will minimize health risks. To protect the health of consumers against the entry of residues of anticoccidial drugs into the food chain, it is necessary to monitor drug residues in animals for food production and for valid veterinary hygienic legislation to pay appropriate attention to this group of drugs (Jevinova *et al.,* 2010). Some anticoccdial drugs such as ionophores are not used in human medicine due to their potent cardiovascular effects. Ensure that recommended withdrawal periods are observed, it has been suggested that residues of ionophores in food could cause adverse health effects in humans as a result of their cardiovascular toxicity. Since poultry litter is extensively applied to land as manure ionophores and their degradation products may readily enter the soil and water environment.

Few studies have been published regarding the environmental fate of ionophores and thus it is difficult to assess their potential impact. Biodegradation studies have indicated that monensin is degradable under aerobic conditions with or without manure and in manure piles within 33 days. Degradation in manure piles under anaerobic conditions was less extensive. It should be assumed that the microbiological activity of soil will be affected, at least initially following application of ionophore containing manure and this may affect nutrient release.

Direct effects on plants are not expected except that an inhibitory effect on apple pollen has been reported for monensin. Ionophores may cause irritation and allergic reaction in humans and protective clothing and dust masks should be used whenever there is a risk of exposure. Alarming human health hazards, the emergence of resistant strains of bacteria in birds and passage of these or other resistant factors via food chain from birds to human beings. Use of antibiotics at sub-therapeutic levels in broiler feeds may lead to the development of resistant strains of bacteria in the bird. While consuming the meat containing residues of antibiotics over protracted period may lead to emergence of resistant gut flora and pathogens in human beings such as *E.coli* and *Salmonella* spp. Production of harmful effects from direct toxicity or from the allergic reactions (hypersensitivity reactions) in persons already sensitized to them.

Some drugs and or their metabolites possess carcinogenic potential e.g.sulphamethazine residues containing meat preserved with sodium nitrate may develop a triazine complex that has a considerable carcinogenic potential. Prolonged ingestion of tetracycline present in the broiler meat has detrimental effects on teeth and bones in growing children. Some tetracyclines, most therapeutic antibiotics are relatively heat stable and *resist* both pasteurization and cooking process (Javaid *et al.,* 2000). Adverse effects on the cartilage development in children may result if the broiler meat contains quinolone residues. Drug residues may destroy the useful micro floraof gastrointestinal tract, especially in children and hence lead to enteritis (diarrhoea, dysentery) like problems. Super infections that refer to as fresh invasion or re40 infection added to an already existing infection. Candidiasis caused by *Candida albicans* is a classical example of the unhealthy consequence of the use

of antibiotics. Residues of chloramphenicol are known to cause bone marrow depression and problems like anaemia in consumers (Javaid *et al.,* 2000).

In addition, there are many safe veterinary drugs and none withdrawal period like, amprolium (Donald and Pharm, 1999). Factors that leading to the occurrence of antibiotics residues in animal products are; failure to observe drug withdrawal period, extended usage or excessive dosages of antibiotics, non-existence of restrictive legislation or their inadequate enforcement, poor records of treatment, failure to identify treated animals, lack of advice on withdrawal periods, off-label use of antibiotics, availability of antibiotics to lay persons as over the counter drugs in the developing countries, the addition of antibiotics as milk preservatives during hauling from the centre of production(villages) to the centers of consumption (cities or factories) and lack of consumer awareness about the magnitude and human health hazards associated with antibiotic residues in the food of animal origin (Javaid *et al.,* 2000).

2.25 Anticoccidial Testing in Birds

Three types of tests are generally used to study anticoccidial drugs in broiler birds. These are; Battery tests: Done 7–14 days, tests with birds in wire cages, Standard grow-out test: Done 6–8 weeks tests on birds in floor pens and Full-scale tests which is done in commercial facilities. Each type has a different objective and value to the investigator for example; the battery test is used most effectively to measure the efficacy of an anticoccidial drug against a variety of field isolates of Coccidia. This is an efficient and relatively inexpensive testing procedure. The floor- pen test is an intermediate testing procedure with a primary goal of providing statistically useful performance data under

controlled conditions. Individually, the predictive value of each test is limited. One cannot, for example, confidently extrapolate performance data in a seven-day battery test to market weight, nor can one predict from a few commercial trials the efficacy of an anticoccidial agent in preventing the lesions of major species of Coccidia. As awhole, when properly conducted, the tests complement one another by providing a comprehensive picture of the efficacy, safety and economic value of an anticoccidial agent (Conway and Mckenzie, 2007).

2.26 Immunity to Avian Coccidiosis

2.26.1 Natural (innate) immunity

The surface layer involved with the digestive, respiratory and reproductive tracts is referred to as epithelium and the underlying tissue is the lamina propria. The combination of these two tissues forms the mucosa. Mucosal membrane is considered as the largest organ system in vertebrates. To protect the body from infection within the mucosal immune systems of the gut, respiratory and reproductive tracts have highly developed lymphoid tissues such as the gut associated lymphoid tissue (GALT) and bronchial-associated lymphoid tissues (BALT). In addition, there are well-developed immunological activities that provide essential protection in the different parts of these systems. Within the gut, there are different immunological requirements in different locations, because of the nature of the different local conditions and the specialized functions within different regions. In mammalian species, the GALT contain more lymphocytes than secondary lymphoid tissues, such as the spleen and lymph nodes. It is likely that this is also the case in avian species.

The mucosal surfaces have a number of common features. Since each forms a major barrier between the external environment and internal milieu, they provide an important portal of entry for pathogens. This is especially the case with the gut and respiratory tract where the continuous movement of external substances, nutrients and air, respectively, and the need to transport or exchange essential molecules across the mucosal surface for organs to function properly and for the animal to remain healthy. Some organisms (mainly bacteria) may reside and have a beneficial effect on digestive processes, while pathogenic organisms can replicate in the mucosal epithelial cells or cross the mucosal surface to enter the body proper and cause disease (Fred *et al.,* 2008). A small-scale, low-density production system can allow a low level of exposure to Coccidia, which permits the chicks to develop immunity without triggering. the disease

However, birds may not pick up enough parasites to cause immunity. In addition, immunity is only species-specific; exposure to one type of Coccidia will not protect a chicken from the other species that can infect it (Rose, 1987). Oral inoculation of *E. tenella* led to parasite invasion of the intestinal caeca and caecal tonsils, protective immunity to *E. tenella* infection produce intestinal lymphocytes and gamma interferon (Chock and Bedford, 1999). Previous applications used vaccination to protect broilers via maternal antibodies, protein complex extracted from gamytocytes stage of *E. maxima* elicited maternal protection and enabled young chicks to exposed *Eimeria* spp. without usual sings and consequences of coccidiosis, protection was heterologous against *E. tenella* and *E.acervulina* as well as against the homologous (Micheal *et al.,* 2003).

2.26.2 Acquired immunity

Acquired immunity to *Eimeria* is even more enigmatic than innate resistance to primary infections. All that can be said with any certainty is that immunity to reinfection with *Eimeria* is remarkably effective and is T cell-dependent and that B cells (antibodies) are not involved in acquired immunity since bursectomized birds (Rose and Hesketh, 1979) and mice lacking B cells (Smith and Hayday, 1998) are perfectly capable of developing immunity to reinfection. It has been proven almost impossible to correlate any immune parameter with immunity to reinfection because the expression of that immunity in experimental settings, at least, is so rapid and efficient.

However, studies using gene knock-out mice have proved extremely useful in determining which factors may play a role. Thus, as for primary infection, CD4 T cells are crucial for immunity to reinfection with *E. vermiformis* (Smith and Hayday, 1998). However, in contrast to primary infection, IFN-g plays no role in this acquired immunity (Smith and Hayday, 1998). On the contrary, some studies demonstrate that CD8 T cells can be used to transfer immunity (e.g. to *E. falciformis*; Pogonka *et al*., 2010) or that depletion of CD8þ T cells can increase, very slightly, susceptibility to *E. vermiformis*. Evidence from poultry experiments (Trout and Lillehoj, 1996) is more difficult to interpret because experiments showing an increase in oocyst excretion in secondary infection of birds depleted of CD8þ T cells did not include a concomitant primary infection control, making it hard to assess how significant the increased oocyst production really was. More, and more sophisticated, analyses of acquired immunity to *Eimeria* are required to resolve the mechanism(s) that are operating.

2.26.3 Maternal immunity

The immune system of young animals is 'uneducated' rendering them more susceptible to infectious disease. Protection against infection during this vulnerable period is provided via transfer of antibodies from mother to young. In chickens, this occurs via the egg yolk; indeed, the ability of hens to transfer remarkable quantities of IgY (IgG) antibodies to their hatchlings has long been appreciated, including in regard to the transfer of antibodies that protect chicks from infection with *E. tenella* (Rose and Long, 1971). In many of the progeny from hens deliberately infected with high doses of *E. maxima*, this maternal immunity can be absolute (i.e. result in the complete absence of oocysts in the faeces of chicks), at least during the first week post-hatching. Maternal antibody levels (in egg yolk or chicks) are correlated with protection. Moreover, maternal immunity induced by *E. maxima* confers partial protection against *E. tenella*, possibly via cross-recognition of conserved proteins (or, at least, epitopes) in different *Eimeria* species an idea lent further credibility by the ability of maternal immunization with conserved macrogametocyte proteins to protect hatchlings against multiple species of *Eimeria* (Wallach *et al.* 1994).

The effectiveness of maternal, antibody-mediated immunity to *Eimeria* appears contradictory to the body of evidence, reviewed above, indicating that antibodies play only a minor role in resistance to *Eimeria*. The protection conferred by antibodies was later demonstrated to be correlated tightly with levels of parasite-specific IgG (Wallach *et al*., 1994). Immune sera can even partially protect highly susceptible T cell-deficient animals (Rose and Hesketh, 1979). Thus, antibodies certainly can protect against *Eimeria* but the effect must be described as variable – from absolute to negligible even if similar immunization regimens are used (Wallach *et al*., 1994). Maternal immunization, however,

does appear to be a phenomenon that can be harnessed to control poultry coccidiosis (Wallach *et al*. 1994).

2.27 Development of Immunity During Coccidiosis

In coccidiosis infection, the chickens react in several ways. Following the ingestion of *Eimeria* oocysts, the non-specific portion of the immune system is antagonistic in the form of low pH, enzymes, and inflammatory reactions. This will limit the number of viable sporozoites that reach the site of infection. When the infection is established, the specific immunity system will become active in the form of specific antibodies and specific cellular immunity (Jeurissen and Veldman, 2002). Brandtzaeg *et al.* (1987) defined three general functions of the specific immune response GALT in the host defence against pathogenic infections, including coccidiosis:

a. Processing and presentation of antigens.

b. Production of intestinal antibodies.

c. Activation of cell-mediating immunity.

The role of the specific antibodies in immunity against coccidial infection is limited, but they are present in the circulation and mucosal secretions. The circulating IgY and the biliary IgA that are specific for coccidial parasites have been detected one week after the infection and reach peak values within 8-14 days and persist for two months (Lillehoj and Ruff, 1987). Lillehoj (1988) reported that bursectomised chickens could show full protection against coccidiosis in the absence of antibodies, illustrating that the role of antibodies is minor in the process of immunity against coccidiosis.

In vitro studies showed that immune sera increased the phagocytosis of sporozoites and merozoites (Onaga and Ishii, 1980; Bekhti and Pery, 1989). It is possible that antibodies reduce the invasion of some, but not all *Eimeria* species, or enhance the intraluminal destruction of the sporozoites if they come into close contact with local antibodies before they enter the host cells (Lillehoj and Trout, 1996). On the other hand, T cells have been reported to play an important role in the immune responses to coccidiosis (Rose and Hesketh, 1982; Isobe and Lillehoj, 1993). Trout and Lillehoj (1995) studied the role of $CD4^+$ and the cytokines produced in coccidiosis infection, and found that depletion of $CD4^+$ cells have no effect on *E. acervulina* infection, but results in a significant increase in oocyst production following *E. tenella* primary infection. The authors suggested that this difference could be related to the changes that occur during these infections or that the immune mechanisms may vary from one gut location to another. In contrast, depletion of $CD8^+$ results in a substantial increase in oocyst production following a challenge with *E. acervulina* infection in chickens. The direct role of the $CD8^+$ T cells in resistance to coccidiosis has not been proven yet. However, increased numbers of these cells were seen, and in direct contact with parasite-infected epithelial cells, in a tissue section of the gut following secondary infection, suggesting that infected epithelial cells may be the target of the cytotoxic T cells (Lillehoj and Bacon, 1991; Trout and Lillehoj, 1995).

2.28 Vaccination Against Coccidiosis

The application of attenuated vaccines for the prevention of chicken coccidiosis has increased exponentially in recent years. In *Eimeria* spp. infections, protective immunity is thought to rely on a strong cell-mediated response with antibodies supposedly playing a

minor role. However, under certain conditions antibodies seem to be significant in protection. Furthermore, antibodies could be useful for monitoring natural exposure of flocks to *Eimeria* spp. and for monitoring the infectivity of live vaccines (Constantinoiu *et al.,* 2008). Western blotting analysis of parasite antigens prepared from the lining of caeca infected with the attenuated strain of *E. tenella* revealed two dominant antigens apparently associated with trophozoites and merozoites that were present at high concentrations between 84 and 132 hours post-infection. When cryosections of caeca infected with *E. tenella* were probed with IgY purified from immune birds the most intense reaction was observed with the asexual stages. Western blotting analysis of proteins of purified sporozoites and third generation merozoites and absorption of stage-specific antibodies from sera suggested that a large proportion of antigens are shared by the two stages. The time-courses of the antibody response to sporozoite and merozoite antigens were similar but varied depending on the inoculation regime and the degree of oocyst recirculation (Constantinoiu *et al.,* 2008).

In the past, most broiler producers have controlled coccidiosis by providing anticoccidial drugs in poultry feed; this approach is becoming less desirable in light of growing public concern about food safety. Vaccination consists of infecting young poultry with a known dose of live coccidian parasites. This vaccination will immunize poultry against the disease (Badran and Lukesova, 2006). Avian coccidia are highly immunogenic and primary infections can stimulate solid immunity to homologous challenges. Therefore, it would seem obvious that vaccines could offer excellent alternatives to drugs as a means controlling coccidiosis.

Live vaccine for coccidiosis control have been used to a limited degree by the poultry industry. For about 60 years, their effectiveness hinges on the recycling of initially very low doses of oocyst and the gradual build up of solid immunity. They have been used primarily to protect breeder and layer flocks. However, their use, particularly in broiler flocks, is increasing. Live vaccine contains attenuated or not coccidial strains. Advantage of attenuated vaccines is that they have low reproductive potentials. This present crowding in the specific mucosal areas of infection. Thereby resulting in the development of optimal immunity with minimal tissue damage. It is believed that the drug-sensitive, attenuated strains and wild, native strains interbred, reducing both virulence and drug resistance in local population. Thus, the useful period of anticoccidial drugs could be extended by rotating their application with live vaccine (Badran and Lukesova, 2006). A low-molecular-weight immunogenic antigen with a single immunodominant epitope was reported to be present in all endogenous stages of *E. tenella.* Metabolic antigens from developing sporozoites, merozoite antigens and gamete antigen all elicit various degree of protective immunity.

A delivery mechanism for coccidial vaccines that produces optimum resistance to challenge infection is not yet determined. Immunogenic *Eimeria* antigens have been administered as isolated proteins with adjuvants as recombinant antigens in live vectors such as non pathogenic strains of *Escherichia coli, Salmonella enteric, serovar and typhimurium*, poxviruses, fowlpox virus and turkey herpesvirus and by direct plasmid DNA injection with various degree of success (Badran and Lukesova, 2006). A species-specific immunity develops after natural infection (Witlock *et al.*, 1975). The degree of

which largely depends on the extent of infection and the number of reinfections. Protective immunity is primarily a T-cell response.

Commercial vaccines consist of low doses of live, sporulated oocysts of the various coccidial species administered at low doses in day-old chicks. Because the vaccine serves only to introduce infection, the vaccine strains of coccidia may or may not be attenuated. The self-limiting nature of coccidiosis is used as a form of attenuation for some vaccines, rather than biological attenuation, Layers and breeders that are maintained on floor litter must have protective immunity. Often, they are given a suboptimal dosage of an Anticoccidial drug during early growth, with the expectation that immunity will continue to develop from repeated exposure to wild types of Coccidia. This method has never been particularly successful because of the difficulty in controlling all of these factors (Marshall *et al.*, 2003). Anticoccidial vaccines may not induce complete immunity in chickens with lowered immunocompetence due to stress, including certain viral diseases (Maan and Bhutani, 1994).

2.28.1 *In ovo* vaccination

Developed by the Poultry Health Division of Pfizer Animal Health, is delivered via *in ovo* administration and will provide a new tool for the broiler industry to help control one of the global poultry industry's most prevalent and costly diseases. *In*-ovocox is administered *in ovo* to 18 or 19 day-old incubated broiler chick eggs via an in ovo injection system.The *in ovo* administration of Inovocox helps ensure that every bird receives a uniform dose for effective protection. This technology is based on more than a decade of research, involving millions of birds to evaluate Inovocox for efficacy and safety.

The Inovocox vaccine contains highly immunogenic, anticoccidial-sensitive, sporulated oocysts of *E. acervulina*, *E. tenella* and two strains of *E. maxima*. These originated from field isolates, which were screened and selected for their ability to help protect against challenge when administered *in ovo*, and for their sensitivity to anticoccidial drugs. Pre-hatch exposure to coccidial organisms will allow birds to develop early immunity to the disease (Bal, 2009). Early and uniform flock immunity to coccidiosis helps provide control of clinical and subclinical coccidiosis and may result in more uniform growth and development of the flock throughout the grow-out. Inovocox has no significant effect on hatch rate. Performance trials show Inovocox-vaccinated flocks will help provide attractive weight gain, feed conversion and settlement costs.

In addition, Inovocox vaccine may be used as a year-round coccidiosis control program, or as part of an annual rotation program. One dose of Inovocox helps provide broiler birds with life-long immunity against coccidiosis, the new vaccine is a useful addition to the use of *in ovo* injection systems, which already is utilised on a large scale in the broiler industry. It seems that in ovocox will be a new convenient, efficient and precise method of coccidiosis protection (Bal, 2009).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

The experimental settings was at the PETCA building, Anguldi, 5 kilometers from the National Veterinary Research Institute, Vom, Jos Plateau State, Nigeria, where the laboratory work was carried out. The Jos Plateau lies on the pre-cambian from the cambian to jurasic northern Nigeria crystalline complex in central Nigeria. Its average elevation is about 1,250 m above mean sea level. The state is bounded on the north and west by Kaduna plains (on the average of 600 m above mean sea level) and on the south by Benue plains (on the average of 700 m above mean sea level), (PADP, 2002). Geographically, the Jos Plateau is located between latitude 08°24'N and longitude 008°32' and 010°38' east. The land surface of Jos Plateau consists of plains, hills, depressions and todes of various forms, shapes and sizes. It is a major tourist centre in Nigeria with agriculture as the main occupation of the people. The high altitude confers on the Plateau lower temperature than those encountered elsewhere in Nigeria except the Obudu and Mambilla Plateau. The dry season is determined by the north easterly tropical continental air masses known as harmattan (from October – April) and the wet season is the most tropical maritime air masses from May – September. The average annual rainfall is about 1,100 mm and is evenly distributed. Another element of climate is temperature December and January experience temperatures of below 15^0C. During February and March, the temperature rises again about 25^0C. Most of the human activities are mining and agriculture involving rearing of chickens in both the rural and urban areas for subsistency and income (PADP, 2002).

3.2 Experimental Birds

Four hundred (400) day-old broilers (marshal breed) were purchased from ECWA farms, Jos, brooded and used for the study. The birds were randomly distributed into six different groups of 40 each, in a clean wire cage (n= 40). At two weeks old, each group was again subdivided into two, treated and non treated, of twenty broilers (n=20) each. The birds were kept in a clean building, and the legs banded or labelled under strict biosecurity measures. Feed (Broiler starter, Grand cereals and oil mills, PLC, Zawan, Jos-Plateau, Nigeria) and water were provided *adlibitum* The birds were vaccinated with Newcastle disease vaccine (La-Sota) at day 21 and Gomboro disease vaccines at days 14 and 28.

3.3 Source and Isolation of *Eimeria tenella* oocysts (Local Isolate)

The oocyst of the parasite was obtained from naturally infected birds from a farm in Jos, Nigeria. The method described by Rose and Long (1980) and as modified by Amer *et al.* (2010) was used for the isolation of unsporulated and sporulated *Eimeria tenella* oocysts. Briefly, the whole intestinal caeca of naturally infected chickens with bloody diarrhea were collected and taken to Helminthology Laboratory of the Parasitology Division, National Veterinary Research Institute, Vom where the caeca were carefully opened up and the caecal contents poured into sample bottle for procession using flotation method to recover oocysts. A slurry of feaces was made by adding 10 parts of water to I part of feaces and subjected to floatation using sugar as the medium. The oocysts were concentrated by centrifugation at 1000 rpm for 5 minutes, and washed in water to obtain unsporulated oocyst. The oocysts were sporulated by incubating for 24-72 hours in 2.5% potassium dichromate. The sporulated oocyst used to orally infect two-week old broilers and their caeca collected and opened to harvest enough quantities of the unsporulated oocysts (by

washing in 5.25% sodium hypochlorite to stop sporulation) and sporulated oocysts (by re-suspending some unsporulated oocysts in 2.5% potassium dichromate and aerated for 24-72hours at room temperature and were stored at 4°C.

3.4 *In vitro* production of Schizonts, Merozoites and Gametocytes

3.4.1 *In vitro* culture and harvesting of schizonts

3.4.1.1 Preparation of bovine monocytes for culture of schizonts

The bovine monocytes were prepared as follows:-

Fifty apparently healthy 8-24 months old male Hereford-Angus heifers were selected at random from the herds of the Experimental Animal Farm, College of Medical Laboratory, National Veterinary Reseach Institute, Vom. Blood samples were collected by jugular venipuncture using needle and tubes containing acid-citrate-dextrose (ACD) as anticoagulant. Mononuclear leucocytes were isolated by modification of the procedure of Birminghan and Jeska (1980). Briefly, the uncoagulated blood was centrifuged at 1,000 g for 20 minutes and the buffy coat layer collected. Contaminating red blood cells were lysed by the addition of 2 volumes of cold phosphate-buffered (0.013M) deionized water, and isotonicity restored with 1 volume of phosphate-buffered saline for 10 minutes after which the cells were washed 4 times with Hank's balanced salt solution (HBSS) without Ca^{2+} and Mg^{2+}. The leucocytes were resuspended in Dulbecco Modified Eagle Medium (DMEM) supplemented with 50 µ/ml gentamycin sulphate, 15% foetal bovine serum (FBS) plus 15% horse serum (HS), the sera were previously heated in a microwave oven (DJ-M017-1, China) at 56^0C for 30 minutes. The cell cultures were incubated at 37^0C and 5% Co_2 in an air-humidified incubator 7 days. The culture medium was changed every 2 days. and on day 7 the culture medium centrifuge at 1,000 g for 20 minutes. The harvested

monocytes in the flasks were used immediately, though occasionally stored at 4 ^{0}C until needed.

3.4.1.2 Inoculation of bovine monocytes with sporulated oocysts for production and harvest of schizonts

Each freshly prepared flask of bovine monocytes was inoculated with 10^3 sporulated *Eimeria tenella* oocysts harvested from the infected experimental broiler chickens maintained in the Protozoology Laboratory, N. V. R. I. Vom, Nigeria. The oocysts were counted in the Mcmaster counting chamber. The cultures were then incubated for 10 days at approximately 37^{0}C in a 5% $CO_{2,}$ incubator with 15ml fresh media added every three days. Thereafter, the culture supernatant and infected cells were harvested by scraping the monolayer into the culture fluid and 1/20% of the culture were inoculated on the monolayer of fresh (uninfected) bovine monocytes at less than 80% confluence. The cultures were observed daily for schizonts released from the host cells and floated in the medium. The viable schizonts were collected by decanting the culture fluids followed by centrifugation at 800 g for 6mins to concentrate the schizonts. The schizonts were used immediately to orally infect the broiler chickens.

3.4.2 Production and harvesting of merozoites

The method of Stotish and Wang (1975), Witlock and Danforth (1982) and Xie *et al*. (1990) were used. Briefly, Four-week-old broiler chickens were used to harvest *E. tenella* merozoites. The birds were orally infecated, each with 10^5 sporulated *E. tenella* oocysts and sacrificed at 108, 120 and 132 hours post inoculation (p.i.). The caeca were opened and the bloody debris or caecal cores were removed by washing in Hank's Buffered Balance solution (HBSS). The mucosal surfaces were scraped with a scapel, and the tissue scraping

were placed in HBSS. These materials were gently filtered through cheese cloth with HBSS (Witlock and Danforth, 1982) and merozoites in the filterate were counted in a haemocytometer.

3.4.3 *In-vitro* production of sporozoites by excystation of sporulated oocyst

Characterized sporulated oocysts of *E. tenella* (local isolates) maintained in the Protozoology Laboratory, Parasitology Division, National Veterinary Reseach Institute (N.V.R.I) Vom, Plateau State, Nigeria were processed for excystation to release sporozoites (Speer *et al*., 1973). Briefly, purified sporulated oocysts were treated with 2.5% sodium hypochlorite for 20 minutes followed by continuous stirring in a vessel containing sterilized glass beads (425-600 µm diameter) for 25 minutes on a magnetic stirrer. The excysted material was centrifuged at 310 g for 10 minutes, the supernatant was discarded to remove sodium hypochlorite, and the sediment was washed three times with phosphate-buffered saline (PBS). The washed sporocysts were suspended in excystation fluid (0.25 g trypsin, 4.0 g taurodeoxycholic acid and 0.094 g magnesium chloride brought to 100 ml volume with HBSS at pH 7.8-8.0) followed by homogenization for 7 times (cycled on/off for 30 minutes on ice) at 40^{o}C and 5% CO_2).The excysted sporozoites were obtained by centrifugation at 310 g for 10 minutes and stored in PBS at 4^{o}C for further use.

3.4.3.1 In vitro production and harvesting of gametocytes

Gametocytes were generated following the protocol of Akhtar *et al*. (2002) and Hafeez (2005). Briefly, three hundred chicken eggs (at 9 days of embryonic development; ED) were procured from a local hatchery in Jos, Plateau State kept and maintained at 39^{o}C and 70% relative humidity in an incubator. Candling was performed to confirm the viability of the embryos at day 12 ED and 0.l ml suspension of sporozoites obtained from the the

excystation of sporulated oocysts of *Eimeria tenella* were inoculated into chicken embryos through the chorio-allantoic membrane along with penicillin (2,000 IU) and streptomycin (0.05 mg). The embryos were maintained at 70% humidity for 5-.7 days (Akhtar *et al.*, 2002). On day 5 -7 post-inoculaton, chorio-allantoic fluid was collected from the dead embryos to harvest the gametocytes (Hafeez, 2005). Gametocytes were concentrated by centrifugation at 1,500 g for 5 minutes and washed twice with sterile PBS and either used immediately or stored in PBS at 4^0C until needed.

3.5 Microscopy of *Eimeria tenella* Developmental Stages

Images of the *Eimeria tenella* developmental stages experimentally generated were taken using the Zeiss Axiorest 100 microscope connected with the cacl zeiss sony cuber shot 12.1 mega pixels monochrome CN50CC camera.

3.6 Experimental infection of broilers with infective materials and monitoring

The experimental birds, except the control were orally given primary and secondary challenge infections with the various developmental stages of *Eimeria tenella*, respectively at week 2 and $3^1/_2$ while at week 5 of age, all birds were infected the sporulated oocyst of the parasite (Table 3.1). Each group was subdivided into Treated (n = 20) and Non-Treated (n = 20). In each infected group, birds in one of the subdivisions were treated with amprolium 250 WSP^R Holland was administered in drinking water at a concentration of 250 mg/1 (0.025%) for a period of 5 days as prescribed by the Manufacturer at the appearance of visible clinical signs.

To obtain serum, blood samples were collected from the experimental birds using the method described by Talebi and Mulcahy (1995). Briefly, 1 ml of blood sample was

obtained from the wing vein of each bird using 20 gauge needle (Becton Dickson co., Plymouth, UK) into a 2 ml vacutainer. Samples were obtained on days 2, 4, 6, 8, and 10 after primary and secondary infections, and on days 5, 7, 11,14, 17, 20 and 24 after tertiary infection (Rose and Hasketh, 1982). The blood which had been allowed to clot for 1 hour at room temperature, was left over night at 4^0C and then centrifuge at 800g for 5 minutes. The serum samples were thereafter heated at 56^0C for 30 minutes to inactivate the compliment before storage at -20^0C. All sera were analyzed with the developed ELISA Triplicate

Table 3.1: Experimental infection of broilers with developmental stages of *Eimeria tenella*.

Group	Treatment and No. of birds	Infection type/ Age of bird		
		I^o /wk 2	2^o /wk $3^1/_2$	3^o /wk 2 challenge with virulent *E. tenella*
I	T(n=20)	10^5 USO	10^5 USO	10^5 SO
	NT(n=20)	10^5 USO	10^5 USO	10^5 SO
II	T(n=20)	10^5 SO	10^5 SO	10^5 SO
	NT(n=20)	10^5 SO	10^5 SO	10^5 SO
III	T(n=20)	10^5 SCZ	10^5 SCZ	10^5 SO
	NT(n=20)	10^5 SCZ	10^5 SCZ	10^5 SO
Iv	T(n=20)	10^5 MRZ	10^5 MRZ	10^5 SO
	NT(n=20)	10^5 MRZ	10^5 MRZ	10^5 SO
V	T(n=20)	10^5 GMT	10^5 GMT	10^5 SO
	NT(n=20)	10^5 GMT	10^5 GMT	10^5 SO
VI		0	0	0

KEY; USO-Unsporulated oocyst
SO- Sporulated oocyst
SCZ- Schizoites
MRZ- Merozoites
GMT- Gametocytes
1^o- primary infection
2^o- Secondary infection
3^o- Tertiary infection
WK-Week
T –Treated
NT – Non treated

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3.7 Molecular Identification of *Eimeria tenella*

3.7.1 Ribonucleic acid (RNA) extraction

Three centimeter-long fragments were excised from the middle portion of the caeca of experimentally infected birds with characterized sporulated oocysts of *Eimeria tenella* (local isolate). They were cut (5 grams) longitudinally to remove intestinal contents. Thereafter, the fragments were stored in ribonucleic acid later (RNAlater) at 4^0C until needed for use. When needed the fragments were washed in ice-cold phosphate buffered saline (PBS) and immediately immersed in TRIZoL solution (life Technologies, Cergy Pontoise, France) for 3 minutes under agitation. This technique allows the extraction of RNA from cells of the upper layer of the mucosa (Laurent *et al*., 2001). The fragments of the caeca were crushed with sterile glass beads in a motar containing PBS and the supernatant was centrifuged at 1,000 g for 10 minutes to removed the PBS. The deposit was transferred to 2 ml Eppendorf tubes and stored at -20^0 C. When needed the deposits in the tubes were removed and kept for 5mins at room temperature, and 0.2 ml of chloroform per 1 ml of TRIzol reagent was added. The sample tubes were vigorously shaken by hand for 15 seconds and kept at room temperature for 3mins. The samples were centrifuged at 1,2000 g for 15 minutes at 4^0C to separate the mixture into a lower red phenol-chloroform phase containing DNA, an interphase, and a colourless upper aqueous phase containing RNA. The RNA from the aqueous phase was precipitated by mixing with isopropanol using 0.5 ml isopropanol per 1 ml of TRIzol reagent used for the initial homogenization. The RNA samples were incubated at room temperature for 10 mins and centrifuged at 1,2000 g for 10 mins at 4^0C. The RNA precipitate formed a gel-like pellet on the side and bottom of the tube. The supernatant was removed and the pellet was washed once with 1 ml of 75% ethanol. The samples were mixed by vortexing and centrifuged at 7,500 g for 5

mins at 4^0C. The supernatant was removed and the RNA pellet was dried in the laminar flow cabinet for 5-10mins. The pellet was dissolved in RNAse-free water (DEPC treated). Total RNA was extracted and treated with DNase I (Fermentas GmbH, st. Leon Rot, Germany) prior to purification using the Nucleospin RNA II kit (Machery-Nagel, Duren, Germany). The RNA extraction was performed according to the Manufacturer's recomendations.

3.7. 2 Reverse transcription-polymerase chain reaction (RT-PCR) on extracted *Eimeria tenella* ribonucleic acid (RNA)

Amplification of full length Etgam 22 cDNA was carried out with cDNA from infected caeca at 137 hours post infection in 25 µl reaction volumes containing 5 μl of the 5 x RT buffer, 0.75 μl of a 0.4mM concentration of each dNTP, 11.75 μl Q solution (Qiagen, Hilden Germany), and 0.5 μl expand high-fidelity DNA polymerase (Roche, Mannheim, Germany) in 5 μ1 1 x high fidelity buffer, 1.0 μl forward primer 5^1 - AATTTGTACCATCGCACACC-3^1 (Unpublished) and 1.0μl reverse primer 5^1 – CGAGCCGTCTGCAATGCACA-3^1 (Unpublished). Thermal program of PCR was as follows: denaturation step at 94°C for 2 min, 35 cycles of denaturation at 94°C for 15 s, annealing at 63°C for 30 s and extension at 12°C for 1 min. A final prolonged extension step at 72°C for 10 min completed the polymerase chain reaction process. Sporozoites excysted from *Eimeria tenella* oocysts local isolate was used for positive control and distilled water used as negative control in polymerase chain reaction. To verify the results, 10 µl of each polymerase chain reaction product was electrophoresed in a 1.5% agarose gel, stained with ethidium bromide and visualized on a UV transilluminator (JY045-3L,

Beijing). The polymerase chain reaction product were identified by size using a 100 base pair ladder.

3.8 Post-infection Monitoring of the Birds

Following infection, the birds were monitored daily for clinical signs, oocyst shedding and post-mortem lesions in the dead and sacrified birds.

3.8.1 Clinical signs

The birds were closely observed for the appearance of visible clinical signs such as bloody faeces, ruffled feathers, huddling and mortality following infection.

3.8.2 Oocyst shedding/counting

The infected birds were monitored daily for oocyst shedding following primary-secondary-tertiary infections. The oocysts were quantified as follows:

Faecal samples were homogenized in a blade grinder and 2.5 ml samples were collected from each suspension. The unsporulated oocysts were diluted in sucrose to 1: 10 to 1:10,000 and were counted microscopically in a Mcmaster chamber. Total oocyst number was calculated as oocyst count x dilution factor x (feacal sample volume/counting chamber volume)

3.8.3 Post-mortem examination for gross lesions

Dead or sacrified birds were examined for lesions of caecal coccidiosis. The broilers were sacrificed by cervical dislocation (CFMV, 2002) at the end of each period of infection. Gross lesions were scored using the criteria set by Johnson and Reid (1970) with scores ranging from 0 (no gross lesion) to 4 (extremely severe lesion) three different individuals. Score 0: no gross lesion. Score 1: A few scattered petechiae are found on the caecal wall,

no thickening of the caecal walls, caecal contents present and appearing normal. Score 2: Lesions more numerous with noticable blood in the caecal contents; caecal wall somewhat thickened, but caecal contents normal. Score 3: Large amounts of blood or caecal cores present; caecal walls greatly thickened; with little if any faecal contents in the caeca. Score 4: Caecal wall greatly distended with blood or large caseous cores; faecal debris lacking or included in cores.

3.9 Determination of Immunity Conferred on Birds By Eimeria tenella Developmental Stages

This was done by measurement of immune bodies using Lymphoproliferation assay or Non-radioactive assay, Flow cytometric analysis and Enzyme linked-immunosorbent assay (ELISA).

3.9.1 Lymphocyte proliferation studies

The lymphocyte proliferation assay is widely used to evaluate cell-mediated immunity in normal and disease states in chickens (Pfohl *et. al*., 1997; Miyamoto *et. al.,* 2002). The spleen collected from each of the sacrified broilers (four per group) at the end of each infection were crushed by pressing on fine mesh Petri dishes containing PBS and glass beads. The suspension was then passed through nylon cell strainer (70 µm; Becton, Dickson, Lincoln Park, NJ). The filterate was centrifuged at 250 g for 10 minutes at 4^{o}C and the sediment containing the spleen cells was collected for the study. One hundred microlitre of splenic cell suspension containing 5×10^{5} cells was placed in each of 96-well sterile culture plate (Corning, NY) containing 100 μl of complete Rose Park Memorial Institute (RPMI) media containing various concentrations (0.1, 1.0, 10 or 20 μg/ml).of

concanavalin A (Con-A, Sigma, MO). The plates were incubated for 48 hours at 37°C in a 5% CO_2, 95% humidity incubator (Ansar *et al*., 1994).

3.9.2 Colorimetric analysis

After 48 hours of culture of the splenic cells of the orally infected broiler chickens, Resazurin or Alamar Blue ™ (Accumed International, Westlake, OH, from Biosource/Tago Immunochemicals, Camars, CA) was added at 20*µl/*well, and absorbance value were read at wavelengths of 570 nm (reduced state) and using an optical density Colorimeter Plate Reader (Molecular Devices, Menlo Park, CA) 24 h after the addition of Alamar Blue. Purple colour was observed on the proliferated lymphocytes (Ansar *et al.*, 1994).

3.9. 3 Flow cytometric analysis

Whole blood (20 µl) from each group of treated and non treated broilers orally infected with the parasite stages was added into a test tube, cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD90.2 (Thy1.2), isotype anti-rat IgG_{2a}, κ control antibodies; and kept in the dark for 15 minutes at room temperature. These stained cells were analyzed using CD_4/CD_4% PARTEC CYFLOW-Cyflow counter 2010, USA (Ansar and Sriranganathan, 1994).

3. 9.4 Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) was performed as described by Rose and Hesketh (1982)**.** The sera was used to determine the antibodies titre values using ELISA *Eimeria* kit (Product code E12V0051, Beijing China). Hay Dottom Nune certified

microtiter plates (Roskilde Denmark) were coated with 50 μ1 of soluble *E. tenella* antigen (sporozoites from sporulated *Eimeria tenella* oocysts)/web at a concentration of 5 μg/ml carbonate buffer (pH 9.6) for 1 hour at 39^0C. The plates were rinsed five times with saline/tween (S/T), and treated with 75 μl of PBS containing 3% BSA, 1% rabbit serum and 0.05% sodium azide for 1 hr at room temperature to block non-specific adsorption. The plates were washed five times with saline/tween (S/T). A 50 μl test serum sample, diluted 1 : 1000 in PBS-T (including 1% rabbit serum and 0.05% sodium azide) was added to each well and incubated for 2 hours at room temperature. The plates were washed 5 times with S/T and 50 μl of 1:1000 dilution of rabbit anti chicken immunoglobulin peroxidase (Pelfreeze Rogers, Arkansas) in PBS-T was added. After 2 hours incubation, plates were washed five times with S/T and freshly prepared substrate solution (2mM OPD 6.15Mm H_2O_2 in 0.1M citrate buffer pH 6.0) was added per well. The enzyme-substrate reaction was stopped after 30 minutes by addition of 100 μl to each well of 2N H_2SO_4. Absorbance were measured at 492 nM (A_{492}) in a Biotele ELISA Reader (Ref S1118170, Multiskan Ex, USA). All serum samples from the experiment were analysed on a single da

3.9.5 Enzyme linked immunospot assay

The method of Ayaz *et al*. (2008) was used. Enzyme-linked immunospot assay (ELISPOT assay) was used to detect IgG or IgY in broilers infected with different developmental stages of *Eimeria tenella* using conjugated goat anti-chicken IgG ELISA kit (Cat. No. E-Ch 000296T, Beijing China). Briefly, the spleen was crushed by pressing on fine mesh Petri dishes containing RPMI-1640 (Sigma, Aldrich Cheme, GmbH, Germany). The suspension was then passed through nylon cell strainer (70µm; Becton, Dickson, Lincoln Park, NJ). The filtrate was centrifuged at 250 g for 10 minutes at 4^oC and the sediment

3.10 Data Analyses

The mean oocyst count, post-mortem mean gross lesion score, antibody titre values and CD_4 count were subjected to statistical analysis using two way Analysis of variance (ANOVA) by SPSS version 20. Data was expressed as (mean ± standard error of the mean (mean ± S.E.M). Values of $p<0.05$ was considered significant.

CHAPTER FOUR

RESULTS

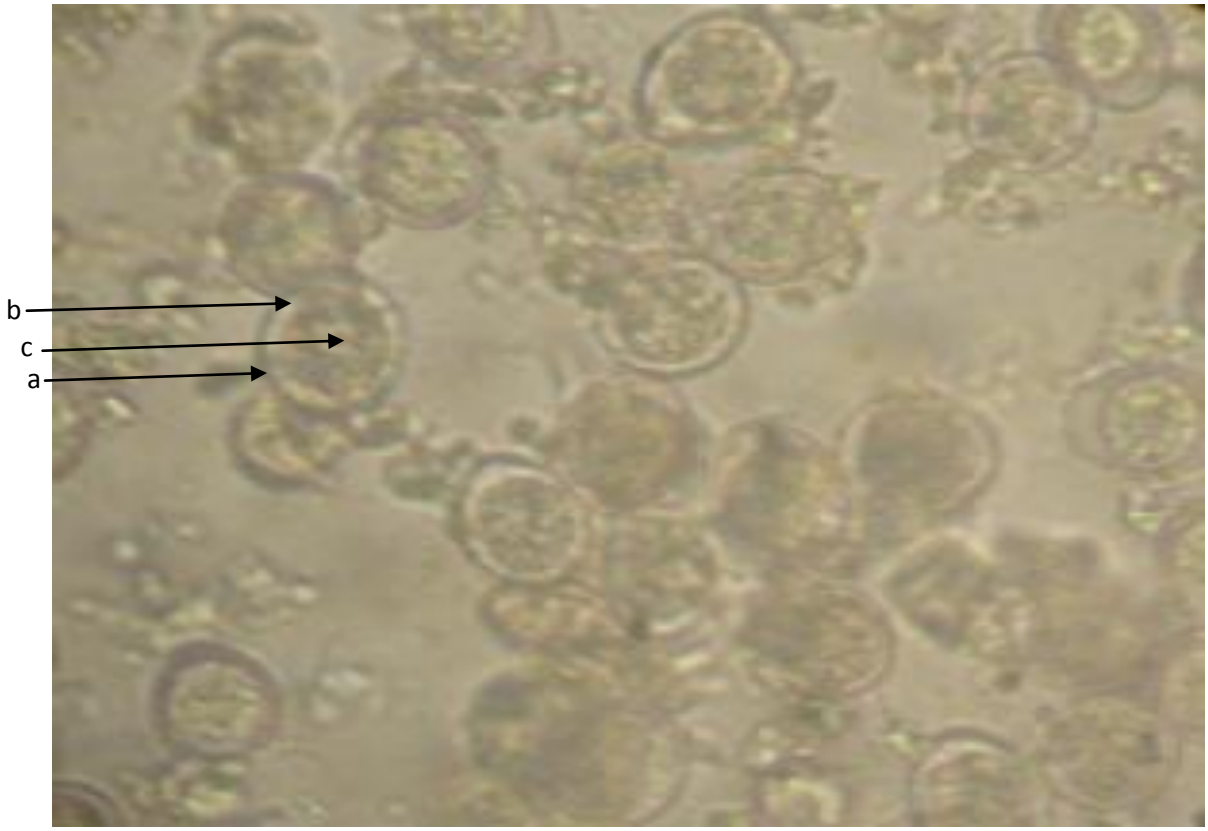


Plate IV: Unsporulated oocyst of *Eimeria tenella* (wet preparation x40), a – outer oocyst wall, b – inner oocyst wall, c - sporoblast

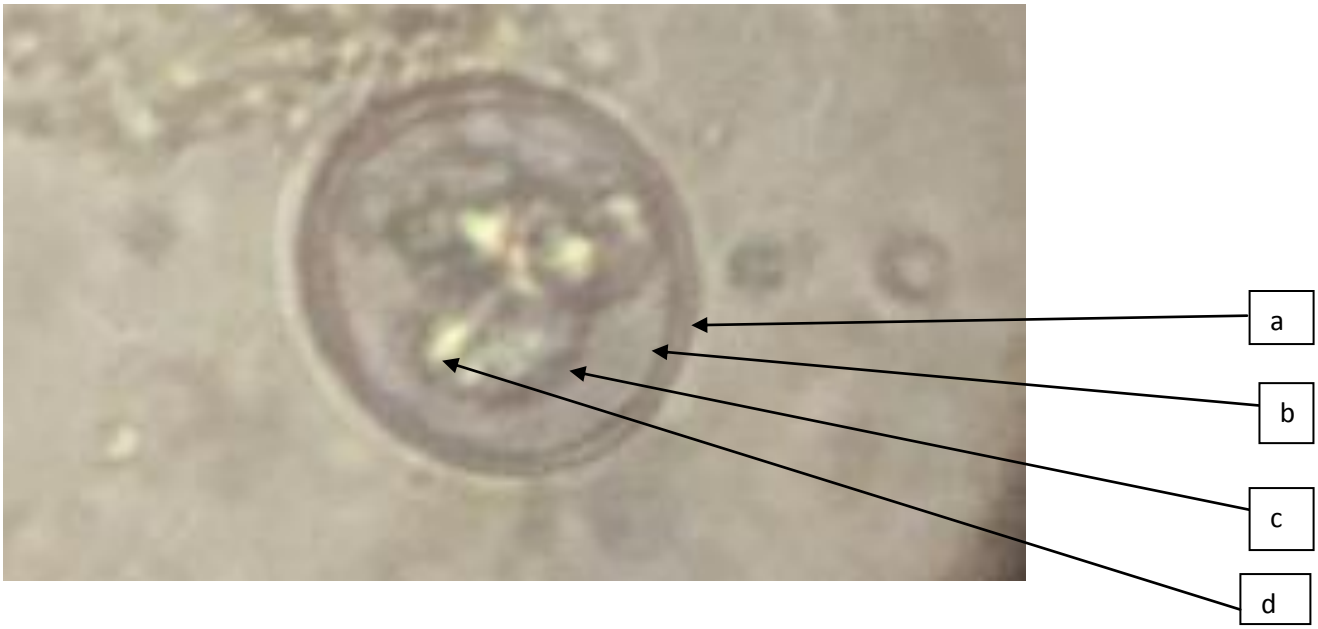


Plate V: Sporulated oocyst of *Eimeria tenella* (wet preparation x40), a- outer oocyst wall, b- inner oocyst wall, c- sporocyst and d- sporozoite

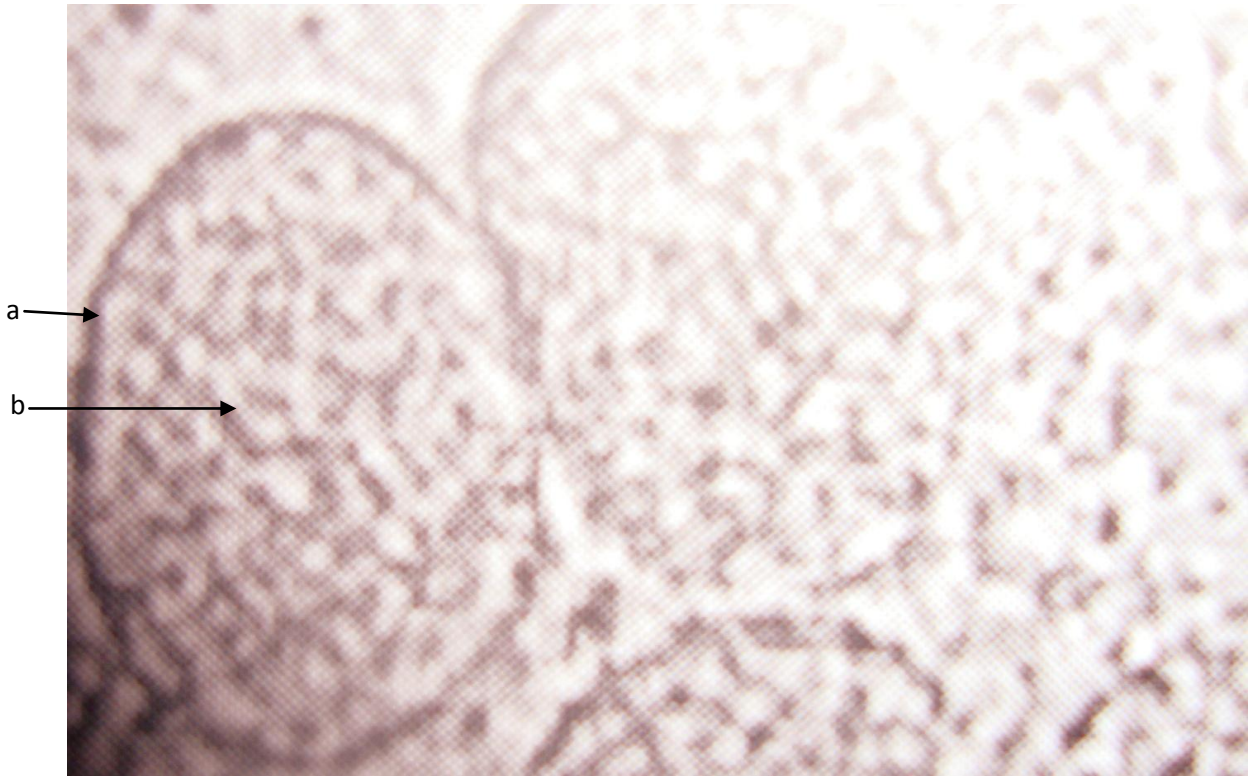


Plate VI: Schizont of *Eimeria tenella* (Wet preparation x40), a – mature schizont, b - merozoite

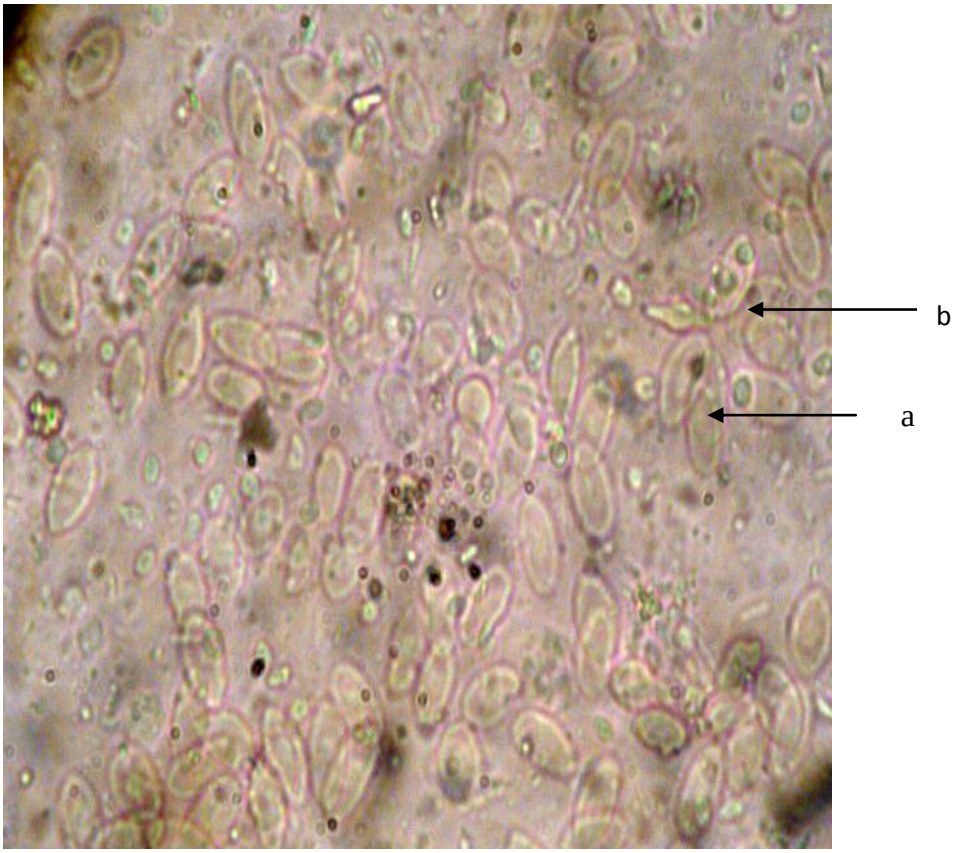


Plate VII: Merozoites of *Eimeria tenella*, (wet preparation x40) a- nuclus, b- pellicle

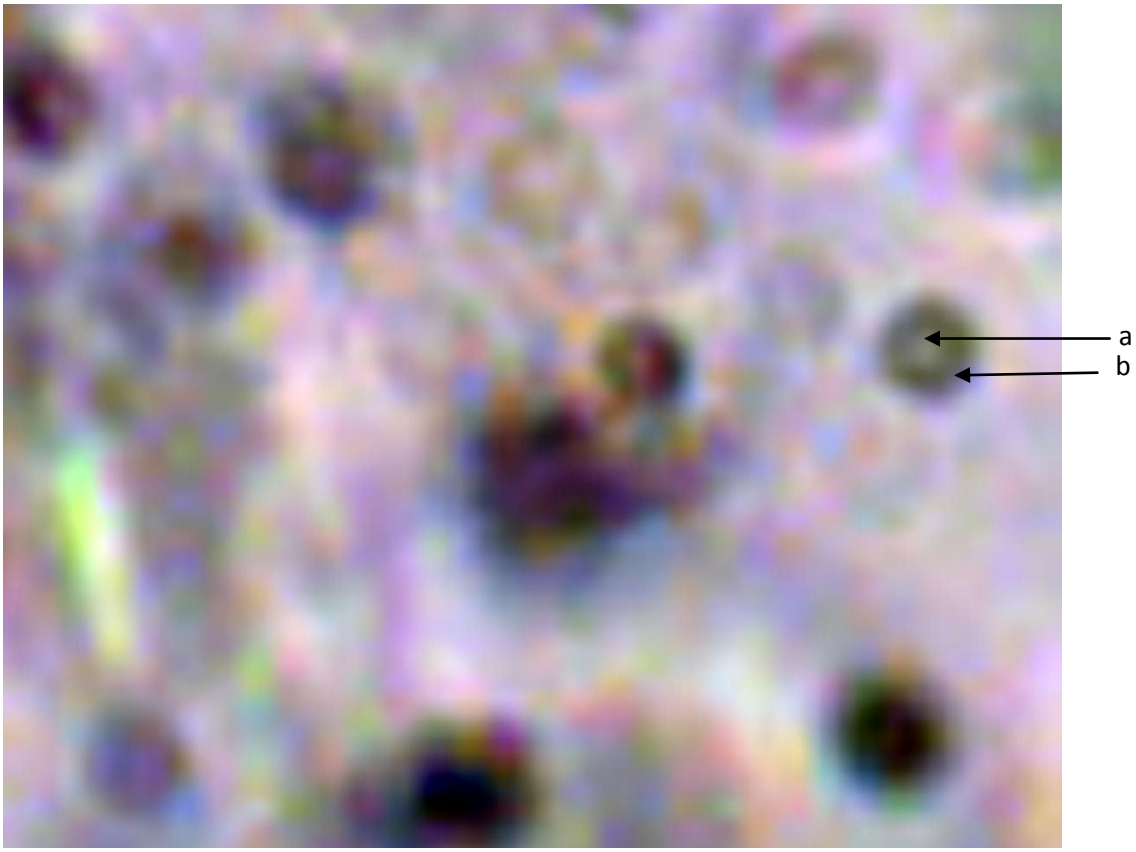


Plate VIII: Gametocytes of *Eimeria tenella* (wet preparation x40), a – macrogamete, b - nuclus

4.3 Clinical Signs in Broilers Experimentally Infected with Different *Eimeria tenella* Developmental Stages

The broilers infected with the unsporulated oocysts and schizonts showed no clinical signs or response in terms of oocyst output, bloody diarrhea or lesions, morbidity and mortality during primary infection (Table 4.1). This situation was also observed at secondary infection when the birds were also infected with unsporulated oocysts. However oocyst output and reddening of serosa of the ceacum were recorded in the experimental broilers infected with schizonts at secondary infection (Table 4.1). Tertiary infection was associated with oocyst output, bloody diarrhea, ruffled feathers in the non treated than the treated birds (Table 4.1) but no mortality was recorded in the broilers infected with the unsporulated oocyst and schizonts throughout the study period (Table 4.5).

The study revealed clinical signs in the non-treated broilers infected with the sporulated oocyst and merozoites characterised by ruffled feathers, huddling, bloody diarrhoea, massive oocyst output count, severe lesions and mortality (Plate IX, Tables 4.1 and 4.2) at primary and secondary infections. At tertiary infection, there was relative decrease in oocyst count (Table 4.2) and gross pathological caecal lesions (Table 4.4) but some of the infected birds recovered. The mean oocysts output or count was 37.07×10^6 in the infected birds non-treated than 25.65×10^6 in the treated groups, although there was a gradual reduction (groups II – 8.36×10^6 – 7.84×10^6 – 5.10×10^6; III - 6.58×10^6 – 4.83×10^6; IV – 7.18×10^6 – 7.00×10^6 – 3.83×10^6; V – 6.59×10^6 – 5.87×10^6 – 4.20×10^6) in oocyst count from primary-secondary-tertiary infections except group I (control), Table 4.2. The treated birds recovered faster from the infection than the non-treated ones. The control broilers showed neither oocyst output, caecal lesions, morbidity and mortality (Plate XI) . There are variations in the mean prepatent period to the different stages of the

parasite used for the experimental infection but the broilers infected with unsporulated oocyst showed none. The prepatent periods of 120, 264, 96 and 48 hours were recorded when the birds were infected with sporulated oocysts, schizonts, merozoites and gametocytes respectively (Table 4.3). There was significant difference ($p<0.05$) in oocyst output of the broilers infected by different stages of the parasite in both treated and non-treated (Tables 4.2).

The non-treated birds recorded the highest mortality from primary – secondary – tertiary infections (4(3.33%)), 3(2.5%) and 6(5.00%) and than the treated birds 1(0.80%), 0(0.00%) and 2(1.67%) respectively. Mortality was common in broilers infected with sporulated oocysts and merozoites 1(5.00%), 2(10.00%), 0(0.00%). 1(5.00%), 1(5,00%), 1(5.00%) treated and non-treated groups indicating that these stages are virulent and initiated caecal coccidiosis in broilers. At tertiary infection mortality occur in all the group except control infected with a known vinular strain of *Eimeria tenella* sporulated oocyst.(Table 4.5).

4.4 Mean Gross Lesions of Caecal Coccidiosis in Broiler Chickens Infected with Various *D*evelopmental Stages of *Eimeria tenella*

All the categories of lesions were observed which included +4 (extremely severe lesion score), +3 (severe lesion score), +2 (moderate lesion score), +1 (mild lesion score) and 0 (no lesion scores) (Plates IX - X). The +4 and +3 lesion scores were prominent in groups II and IV that were infected with sporulated oocysts and merozoites respectively at primary infection (p.i.) and the +2 and +1 scores in groups III and V at post infection. and no score in groups I at both p.i. and secondary infection (p.s.i.) respectively (Tables 4.5). At secondary infection, +3 and +2 lesion scores were observed in groups II, III, IV and V.

Tertiary infection of the broilers showed lesion scores of +2 and +1 in groups II, III, IV and V and +3 in group I. The study recorded a mean lesion score of below +3 in the experimental broilers except group II of the not treated at primary infection (Table 4.4) recorded 3.50 and the lowest mean lesion score of 1.25 in the treated groups II, IV,and V at secondary and group IV at tertiary infections respectively (Tables 4.4). There was significant difference in the lesion scores of the study birds ($P<0.05$).

Table 4.1 Clinical signs in the experimentally infected broilers with the developmental stages of *Eimeria tenella*

Group and treatment	Clinical signs/Infection periods		
	1^0	2^0	3^0
I	USO	USO	SO
T			Bloody feaces ruffled feathers huddling oocyst shedding
NT			Bloody feaces ruffled feathers huddling oocyst shedding mortality
II	SO	SO	SO
T	Bloody feaces Rough feathers Huddling Oocyst shedding	Oocyst Shedding Rough feathers Huddling	Oocyst Shedding
NT	SO Bloody feaces Ruffled feathers Huddling Oocyst shedding	SO Bloody feaces Ruffled fealthers Huddling OOcyst shedding	SO Bloody feaces ruffled feathers huddling oocyst shedding Mortality
III	SCZ	SCZ	SO
T		Diarrhoea Huddling Oocyst Shedding Diarrhoea	Diarrhoea Huddling Bloody feaces Ruffled feathers
NT		Huddling Oocyst shedding	Huddling Oocyst Shedding
IV	MRZ	MRZ	SO
T	Bloody feaces Ruffled feathers Huddling Oocyst Shedding	Oocyst shedding Rough feathers Huddling	Oocyst Shedding
NT	Bloody feaces Rough feathers Huddling Oocyst Shedding	Bloody feaces Ruffled feathers Huddling Oocyst shedding Mortality	Bloody feaces Rough feathers Huddling Oocyst shedding Mortality
V	GMT	GMT	SO
T		Oocyst shedding	Diarrhoea Rough feathers Huddling Oocyst shedding
	NT	Oocyst shedding	Bloody feaces ruffled feathers Huddling Oocyst shedding

Key: USO-Unsporulated oocyst
SO-Sporulated oocyst
SCZ-Schizont
MRZ-Merozoite
GMT-Gametocyte
1^0-Primary infection, 2^0-Secondary infection, 3^0-Tertiary infection
T-Treated, NT-Non Treated.

Table 4.2: Mean oocyst output (10^6) in the broilers infected with the different developmental stages of *Eimeria tenella* treated and non-treated.

Group	Developmental stage	Primary infection		P Value	Developmental stage	Secondary infection		P Value	Developmental stage	Tertiary infection		P Value
		T (n=20)	NT (n=20)			T (n=20)	NT (n=20)			T (n=20)	NT (n=20)	
I	USO	-	-		USO	-	-		SO	2.86 ± 0.29^{d}	2.51 ± 0.34^{a}	0.001
II	SO	3.89 ± 0.43^{a}	4.47 ± 0.76	0.947	SO	3.89 ± 0.38^{c}	3.20 ± 0.58^{b}	0.776	SO	$2.66\pm0.40^{c,d}$	2.44 ± 0.34^{a}	0.002
III	SCZ	-	-		SCZ	3.38 ± 0.32^{a}	3.20 ± 0.58^{b}	0.090	SO	$2.79\pm0.35^{c,d}$	$2.03\pm0.51^{a,b}$	0.001
IV	MRZ	3,34±0.64	3.84 ± 0.72	0.459	MRZ	3.54 ± 0.24^{b}	3.46 ± 0.19^{b}	0.005	SO	2.20 ± 0.31^{a}	1.63 ± 0.48^{b}	0.001
V	GMT	2.54 ± 0.26	3.33 ± 0.28	0.003	GMT	3.24 ± 0.20^{a}	3.34 ± 0.35^{b}	0.051	SO	$2.42\pm0.36^{b,c}$	1.78 ± 0.55^{b}	0.0003

Groups with the same superscripts are not significantly different from each other.
Groups with the different superscripts are significantly different from each other.
Treated and non treated comparison shows a significant difference in groups II and IV

.

Key: USO – Unsporulated oocyst
SO – Sporulated oocyst
SCZ – Schizont
MRZ – Merozoites
GMT – Gametocyte
T – Treated
NT – Non treated

Table 4.3: Mean prepatent period of various developmental stages of *Eimeria tenella* in the experimental broilers.

Groups	*Eimeria tenella* developmental stages	Dose per chick	Prepatent period(hrs)
I	Unsporulated oocyst	10^5	0
II	Sporulated oocyst	10^5	120
III	Schizonts	10^5	264
IV	Merozoites	10^5	96
V	Gametocytes	10^5	48
VI	Control	0	0

Table 4.4 : Post-mortem mean lesion score in the experimental birds infected with *Eimeria tenella* developmental stages.

	Developmental stages used for infection	Primary infection		Secondary infection		Tertiary infection	
		T	NT	T	NT	T	NT
I	Unsporulted oocyst	0.0± 0.0	0.0± 0.0	0.0± 0.0	0.0± 0.0	2.25± 1.25	2.50±1.29
II	Sporulated oocyst	2.50± 1.00	3.50± 0.58	1.25± 0.50	2.00± 0.82	1.25± 0.96	1.50± 1.29
III	Schizonts	0.0± 0.0	0.0± 0.0	0.0± 0.0	0.0± 0.0	1.75± 0.96	2.75± 1.26
IV	Merozoites	1.75± 0.50	2.50± 0.58	1.25 ±0.50	1.50 ±0.58	1.50± 0.50	1.50 ±1.29
V	Gametocytes	0.0± 0.0	0.0± 0.0	1.25± 0.50	1.75 ±0.96	2.00 ±0.82	2.25 ±1.71
VI	0.0	0.0	0.0	0.0	0.0	0.0	0.0

The result showed that the mean gross lesion score was significantly higher in groups II and IV as compared to other groups ($p < 0.05$) for the treated and the non treated at primary infection. A comparison of the treated and non treated, showed no significant difference in groups II and IV ($p > 0.05$) respectively at secondary infection. The result showed that there was no significance in mean gross lesion score in groups II, IV and V when compared with groups I and III for both treated and non treated ($p > 0.05$). Lesion score 0 to +4.

Table 4.5 : Mortality rate (%) recorded in the broiler chickens infected with various developmental stages of *Eimeria tenella* treated non-treated.

Group	Developmental stage	Primary Infection		Developmental stage	Secondary infection		Developmental stage	Tertiary infection	
		T (n=20)	NT(n=20)		T (n=20)	NT (n=20)		T (n=20)	NT (n=20)
I	USO	0(0.00)	0(0.00)	USO	0(0.00)	0(0.00)	SO	0(0.00)	2(10.00)
II	SO	1(5.00)	2(10.00)	SO	0(0.00)	1(5.00)	SO	1(5.00)	1(5.00)
III	SCZ	0(0.00)	0(0.00)	SCZ	0(0.00)	1(5.00)	SO	0(0.00)	1(5.00)
IV	MRZ	0(0.00)	1(5.00)	MRZ	0(0.00)	1(5.00)	SO	1(5.00)	1(5.00)
V	GMT	0(0.00)	1(5.00)	GMT	0(0.00)	0(0.00)	SO	0(0.00)	1(5.00)
VI	CONTROL	0(0.00)	-	-	-	-	-	-	-
	TOTAL	1(0.80)	4(3.33)		0(0.00)	3(2.50)		2(1.67)	6(5.00)

4.5 Lymphoproliferation Studies in the Experimental Broiler Chickens

Lymphoproliferation assay showed the proliferation of protective cytokines in the spleen of broilers infected with the various developmental stages of the parasite. Groups I (infected with unsporulated oocyst) and III (infected with schizonts) did elicit lymphocytes proliferation (IFN- γ, IL-1, IL-2, IL-4, IL-6 and TNF) at primary and secondary infections respectively. Groups II and IV revealed the proliferation of Interferon (IFN- γ), Interleukins (IL-1, IL-2, IL-4, IL-6), Turmor necrotic factor (TNF), Transforming growth factor (TGF), respectively at primary-secondary-tertiary infections, while Group V (infected with gametocytes) showed IL-1, IL-2 and IL-4 at primary and secondary infections. However, groups I, III and V showed proliferation of IFN- γ, IL-1, IL-2, IL-4, IL-6, TNF and TGF respectively at tertiary infection. The control showed no lymphocytes proliferation.

In the study, the number of CD_4 count increased post infection in treated and non-treated broilers orally administered with various developmental stages of the parasite, reaching a peak at day 10 ((groups I – 198.0 x 10^3 µl, 165.3 x 10^3 µl; 200.0 x 10^3 µl, 156 x 10^3 µl and 196.7 x 10^3 µl, 173.3 x 10^3 µl ; II – 199.0 x 10^3 µl, 186.0 x 10^3 µl ; 197.0 x 10^3 µl, 192.7 x 10^3 µl and 200.0 x 10^3 µl, 194 x 10^3 µl; III – 198 x 10^3 µl, 153.3 x 10^3 µl ; 200.0 x 10^3 µ,l 160.0 x 10^3 µl and 188.7 x 10^3 µl, 166.7 x 10^3 µl ; IV – 193.3 x 10^3 µl, 183 x 10^3 µl; 198.7 x 10^3 µl, 183.3 x 10^3 µl and 190 x 10^3 µl , 188.0 x 10^3 µl ; V – 200.0 x 1 0^3 µl, 198.0 x 10^3 µl ; 187.3 x 10^3 µl , 174 x 10^3 µl and 188.7 x 10^3 µl, 175.3 x 10^3 µl respectively) of primary and secondary infections and day 24 of tertiary infection (Tables 4.2, 4.3 and 4.4). There was significant difference in the CD_4 cell count among different groups of the experimental broilers ($p<0.05$). CD_4 levels were higher in the treated than

the non treated broilers at primary infection (Figure 4.6). The levels of CD_4 cells increases rapidly in the non treated birds at secondary infection, showing a non significant difference in the CD_4 levels in all the groups treated and non treated (Figure 4.7). Groups II and IV of the non treated birds had higher CD_4 levels than groups I, III and V at both secondary and tertiary infections (Figures 4.2 and 4.3). There was significant difference in CD_4 subset between groups of the study birds ($p<0.05$). The current study observed a relationship between the different developmental stages of the parasite and immune responses (humoral and lymphocytes responses). Sporulated oocysts(sporozoites) and merzoites yielded high output in oocysts, antibodies and CD_4 T- lymphocyte numbers than the other groups, throughout the experiment periods (Tables 4.6, 4.7 and 4.8).

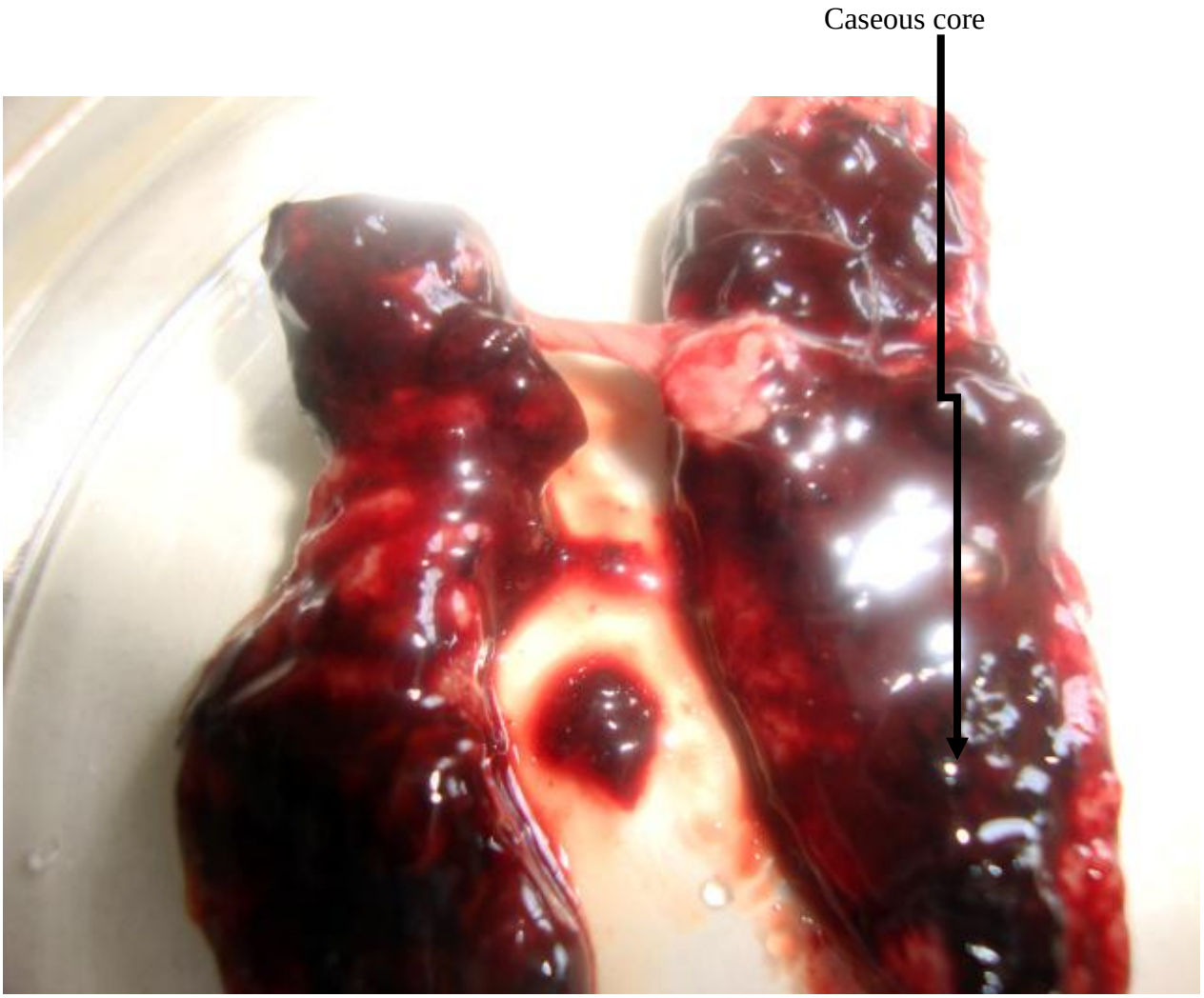


Plate IX: Caecum of a broiler in group II infected with 10^5 *Eimeria tenella* sporulated oocyst showing ballooning and haemorrhagic ceacal plug in the lumen at slaughter after primary infection. (lesion score +4)



Plate X : Ceacum of uninfected broilers (control) showing normal morphology (lesion score 0)

Table1 4.6 : CD_4 Lymphocytes subset counts (cells /10^3 µl)) in experimental broilers orally infected with *E. tenella* developmental stages at primary infection treated and non treated

Group and	Time (days)				
Non treated	2	4	6	8	10
I	133.3±58.6	158.3±61.1	143.3±57.7	133.3±61.1	163.3±57.7
II	120.0±65.6	180.0±58.9	139.3±57.7	131.3±60.8	186.0±85.4
III	143.3±84.7	147.3±84.7	140.3±85.8	133.3±85.5	153.3±76.4
IV	126.7±55.1	166.0±50.7	110.7±53.5	146.0±63.9	183.7±90.2
V	144.0±95.5	170.0±85.4	146.7±76.4	153.3±68.1	180.0±85.4
VI	134.7±102.5	198.0±86.6	195.0±86.6	198.0±86.6	198.0±86.6
Treated					
I	198.7±91.4	200.0±117.9	160.0±52.9	195.3±95.1	199.0±95.4
II	146.0±87.0	200.0±86.8	162.7±83.6	168.0±95.4	199.0±95.4
III	150.0±90.4	200.0±86.6	160±96.0	166.7±83.3	198.0±86.6
IV	163.0±88.9	198.7±94.3	171.0±86.6	177.0±88.9	193.3±90.7
V	135.0±91.7	200.0±91.7	155.3±96.5	169.7±99.9	200.0±86.6

No significant difference was observed across the days for both treated and non treated ($p>0.05$). However, comparison of treated and non treated showed significant difference ($p<0.05$) for all the days.

Table 4.7 : CD_4 Lymphocytes subset counts (cells /10^3 µl)) in experimental broilers orally infected with *E. tenella* developmental stages at secondary infection treated and non treated

Group and non treated	days 2	4	6	8	10
I	140.0±60.8	144.0±62.9	140.0±40.0	154.0±64.1	156.7±70.1
II	183.3±82.5	185.3±86.0	187.3±90.7	189.3±95.0	192.7±84.7
III	143.3±62.5	145.3±63.6	146.7±64.3	170.0±50.7	160.0±69.3
IV	180.0±85.4	168.0±76.9	178.7±81.0	177.3±86.7	183.3±85.8
V	166.7±76.4	170.7±77.1	172.0±76.9	166.0±73.5	174.0±78.1
VI	183.3±104.1	200.0±86.6	200.0±86.6	200.0±86.6	200.0±86.6
Treated					
I	200.0±86.6	196.0±99.1	190.0±96.4	199.3±95.3	200.0±86.6
II	196.7±95.2	188.7±90.3	194.0±91.2	197.3±95.0	197.0±96.0
III	200.0±86.6	192.7±90.7	196.0±92.6	200.0±86.6	200.0±86.6
IV	193.3±95.0	195.3±95.1	196.0±95.2	196.0±91.1	198.7±94.5
V	180.0±85.4	184.0±85.9	186.0±83.1	182.0±81.6	187.3±81.8

No significant difference was observed across the days for both treated and non treated groups ($p>0.05$). However, comparison of treated and non treated showed significant difference ($p <0.05$) for all the days.

Table 4.8 : CD_4 Lymphocytes subset counts (cells /10^3 µl)) in experimental broilers orally infected with *E. tenella* developmental stages at tertiary infection treated and non treated

Group and Non treated	Time (days)						
	5	7	11	14	17	20	24
I	133.3±57.7	140.0±60.8	140.0±69.3	156.7±67.0	160.7±70.4	169.3±76.8	173.3±77.7
II	193.3±90.7	194.7±95.6	194.7±77.1	186.0±88.5	194.0±87.8	191.3±93.6	194.3±85.6
III	165.3±56.6	166.0±28.6	134.0±58.0	168.7±46.3	138.7±46.3	151.3±66.9	166.7±76.4
IV	186.7±90.2	180.7±85.9	189.3±82.4	174.3±79.3	171.0±78.6	174.0±81.3	188.0±90.3
V	140.0±60.8	146.7±64.5	148.0±64.5	171.3±79.1	191.0±41.4	174.0±77.8	175.3±81.1
VI	200.0±86.6	200.0±86.6	200.0±86.6	200.0±86.6	200.0±86.6	200.0±86.6	200.0±86.6
Treated							
I	194.0±95.5	195.3±86.2	196.7±95.2	198.0±95.3	197.3±98.0	192.7±90.7	196.7±89.5
II	196.3±87.4	199.3±95.3	214.0±68.2	196.0±84.9	198.7±90.8	197.3±98.0	200.0±86.6
III	186.7±81.4	178.7±94.2	190.7±88.9	194.0±87.8	166.7±76.4	165.3±71.8	188.7±90.3
IV	194.0±87.8	195.3±95.1	198.0±97.5	170.7±77.1	180.7±85.9	186.7±81.4	190.7±97.0
V	182.0±83.1	180.7±85.9	179.3±79.2	206.0±45.9	184.7±86.3	154.0±66.9	188.7±81.7

No significant difference was observed across the days for both treated and non treated ($p>0.05$). A comparison of treated and non treated showed significant difference ($p<0.05$).

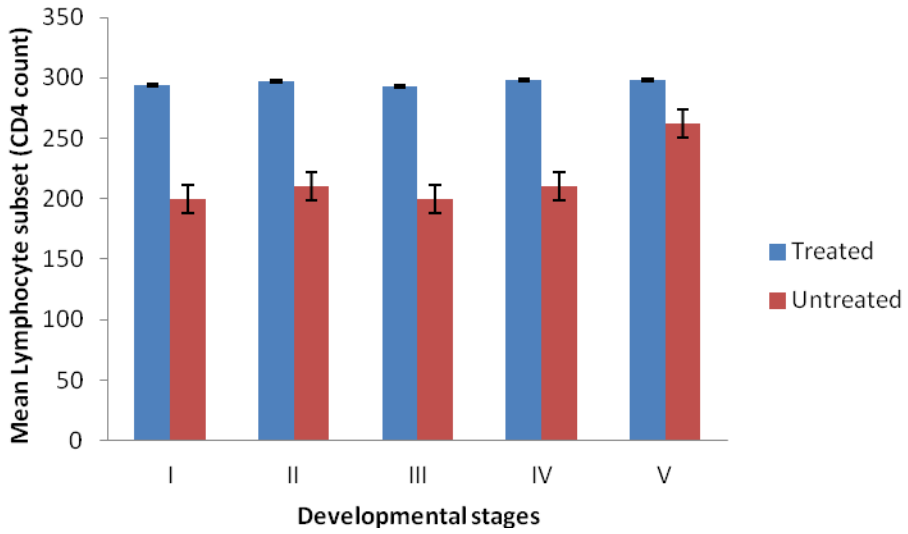


Figure 4.1: CD_4 levels in the plasma of broilers treated and non treated infected with the developmental stages (unsporulated oocyst, sporulated oocyst, schizonts, merozoites and gametocytes) of the parasite at primary infection.

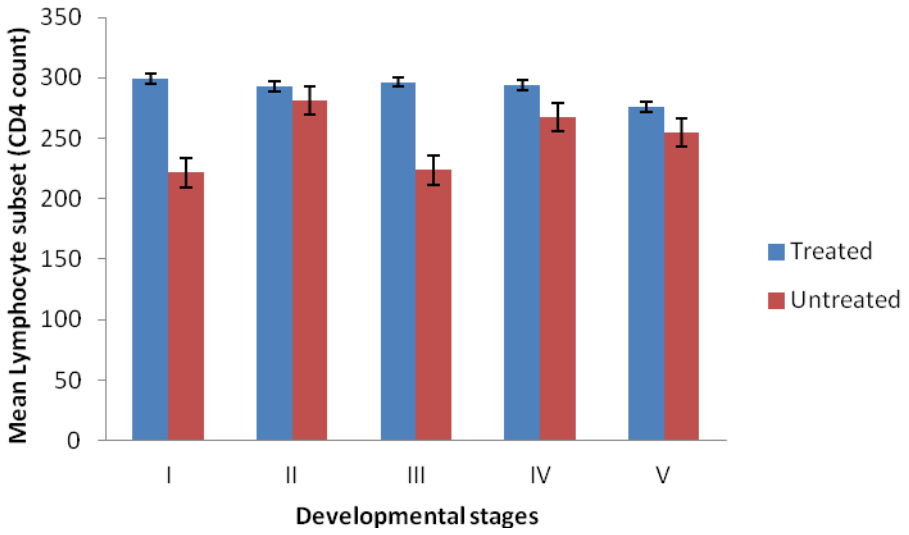


Figure 4.2: CD_4 levels in the plasma of broilers treated and non treated infected with the developmental stages (unsporulated oocyst, sporulated oocyst, schizonts, merozoites and gametocytes) of the parasite at secondary infection.

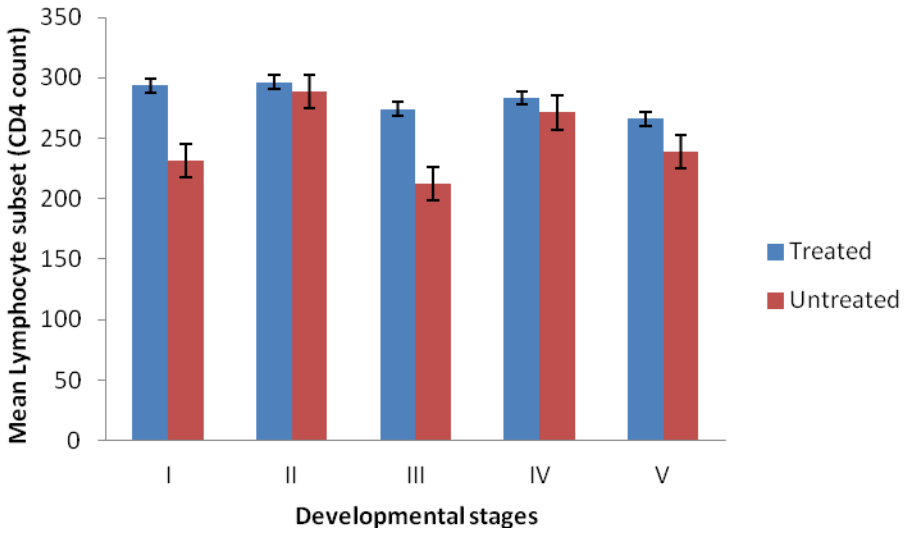


Figure 4.3: CD_4 levels in the plasma of broilers treated and non treated infected with the developmental stages (unsporulated oocyst, sporulated oocyst, schizonts, merozoites and gametocytes) of the parasite at tertiary infection.

4.6 Antibody Response in Broiler Chickens Infected with Different Developmental Stages of *Eimeria tenella*

Antibodies (IgG or IgY) titre values were higher in sera from broilers infected with sporulated oocyst (0.265 ± 0.010, 0.282 ± 0.005; 0.305 ± 0.002, 0.316 ± 0.010 and 0.252 ± 0.002, 0.281 ± 0.010) and merozoites (0.177 ± 0.001, 0.186 ± 0.003; 0.135± 0.010, 0.141 ± 0.002 and 0.069 ± 0.004, 0.139 ± 0.005) reaching a peak on day 10 of post primary and secondary infections and day 5 post tertiary infection in both broilers treated and non treated, (Appendices I, II and III). The antibodies values were relatively low in broilers infected with unsporulated oocysts, schizonts and gametocytes at primary and secondary infection in both treated and non treated broilers at day 10 (Appendices I and II). At tertiary infection, antibodies increases at day 5, 7, 11 and 14 (Appendix I) respectively. Generally, antibodies levels of sera from the infected broilers with the different developmental stages of the parasite, treated and non treated increased post inoculation and after reaching peak levels, they began to decline (Appendix III). The control birds show no antibodies in the sera.. The study demonstrates a non significant difference in the antibody titre values of the treated and non treated sera of the infected broilers groups (II and IV), $p<0.05$ (Appendices I, II and III).

However, the study observed that oocyst output was related to CD_4 lymphocyte subset count, antibody and cytokine productions . Groups II and IV gave the highest number of oocysts than the other groups in both the treated and non treated groups. The study indicates that the higher the number of oocysts the higher the count of CD_4 cells, antibody output and proliferation of cytokines in the blood, sera and spleen of the experimental broilers which were more prominent in broiler groups II and IV.

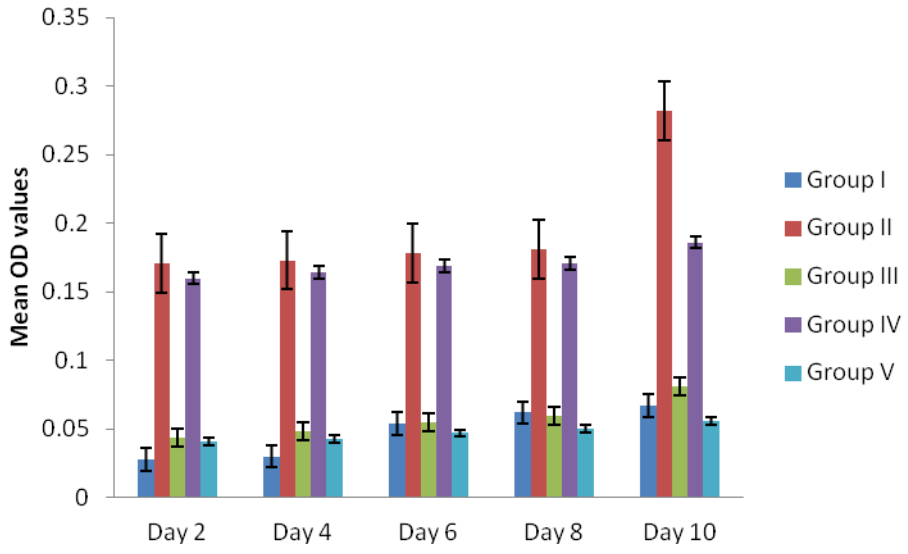


Figure 4.4 : Antibodies level in sera of the experimentally infected broilers with the different stages (unsporulated oocyst, sporulated oocyst, schizonts, merozoites and gametocytes) of *Eimeria tenella* at optical density (O.D) or absorbance of 492 nm) at primary infection.

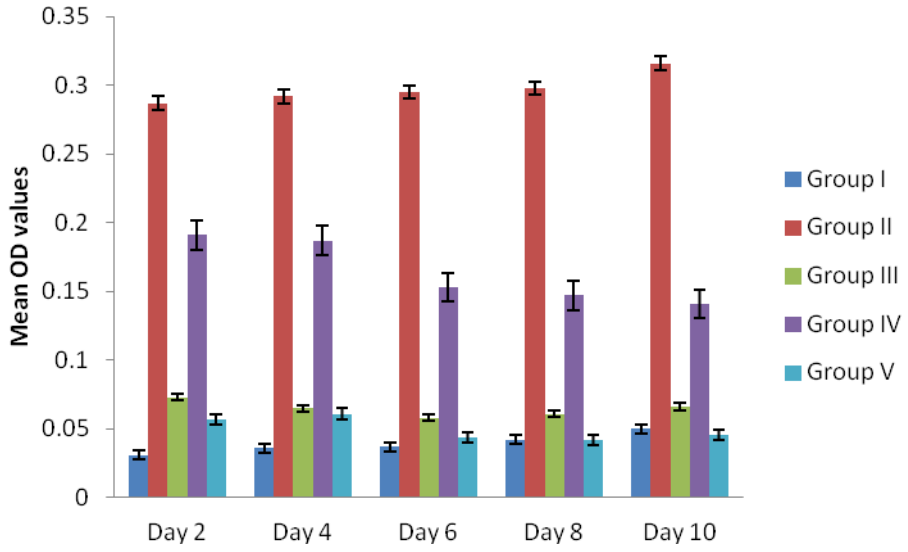


Figure 4,5 : Antibodies level in sera of the experimentally infected broilers with the different stages (unsporulated oocyst, sporulated oocyst, schizonts, merozoites and gametocytes) of *Eimeria tenella* at optical density (O.D) or absorbance of 492 nm) at secondary infection.

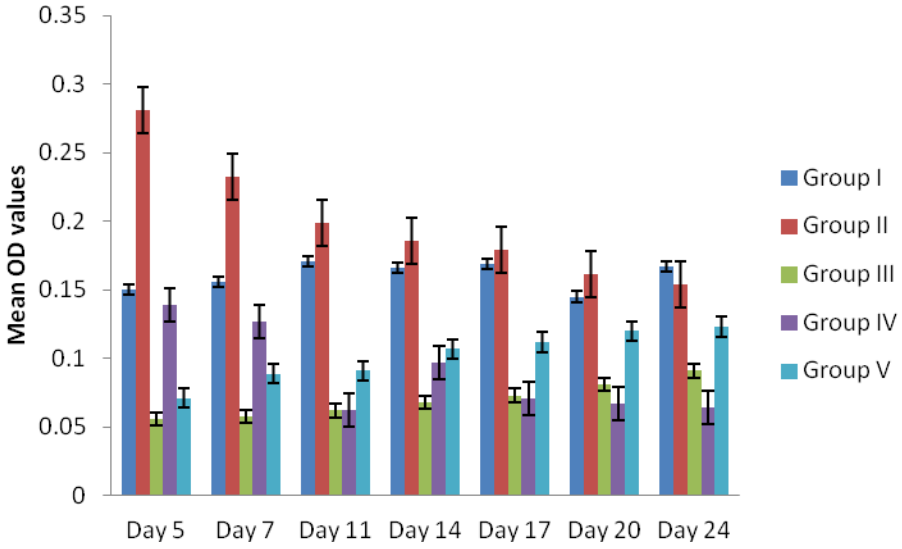


Figure 4.6 : Antibodies level in sera of the experimentally infected broilers with the different stages (unsporulated oocyst, sporulated oocyst, schizonts, merozoites and gametocytes) of *Eimeria tenella* at optical density (O.D) or absorbance of 492 nm) at tertiary infection.

CHAPTER FIVE

DISCUSSION

The exo and endogenous stages of *Eimeria tenella* development, host invasion, nutritional status of the infected broilers and genetic background of the host may play key roles in immune responses to the complex Coccidia. The present study produced the various developmental stages involved in the life cycle of *Eimeria tenella* in the experimental birds. This is similar to the study of Randal (1985) and Woltgang *et al*. (1999) who also generated the different stages in the life cycle of the parasite. Clinical signs were observed in the experimental birds comprising of bloody feaces, ruffled feathers, huddling, and mortality and these clinical signs were similar to those reported by Conway and Mckenzie (2007).

The observed prepatent periods associated with the infection for the unsporulated oocyst, sporulated oocyst, schizonts, merozoites and gametocytes which were 0, 120, 264, 96 and 48 hours respectively are similar to the reports of Assisi *et al*. (2012) who also recorded 120 hours prepatent period for birds infected with sporulated oocyst of *Eimeria tenella*. However the prepatent periods are inconsistent with the other stages of the parasite. This may be due to differences in the potential of the parasite stages to multiply, initiate and complete cycle of development.

There was variation in the oocyst output of the broilers infected with the various developmental stages of the parasite indicating the differences that may exist in the binding potentials of the various stages and is consistent with the reports of Talebi and Mulcahy (1995) who made similar observations in *Eimeria maxima* infection in chickens. This variation may be due to differences in the total number of potent sporozoites released from

the sporocysts, and merozoites released by the schizonts, effect of caecal content on the sporozoites and merozoites, as well as the age and genetic background of the birds (Talebi and Mulcahy, 1995). This indicated that the broilers immunologically responded to the developmental stages of the parasites at different intensities or time, resulting in varied oocyst output. The observed gradual reduction in oocyst output in the treated and non treated experimental broilers from primary- secondary - tertiary infections is similar to the report of Assisi *et al*. (2012). These authors reported that this may be due to the stimulation and production of immune bodies (T and B cells) in the non treated broilers at primary and secondary infections and action of Amprolium in the treated chickens. This is also colloborated by the findings of Chapman *et al.* (2005).

Macroscopic lesions varied for the different periods of infection in the experimental birds. There is presently no consensus on what levels of lesions be considered clinical (requiring treatment) and subclinical (Maarten 2013). However, some consider lesion higher than 1.50 per species as indicative of clinical disease, and levels below as subclinical. However, the present study recorded a mean lesion score in the experimental broilers below +3(severe) except at primary infection were 3.50 was scored in the non-treated broilers in group II and the lowest mean lesion score of 1.25 in the treated birds in groups II, IV and V at secondary and IV at tertiary infections, respectively. This is in agreement with Kadhim (2014) who recorded a mean lesion score of 3.20 for challenged broilers but inconsistent with the study of Raman (2011) who observed lesion score below +2 in chickens. These variations may be due to age, genetic background of the broilers, dosage of infection, stages of the parasites administered and the environment.

The non-treated birds recorded the highest mortality from primary – secondary – tertiary infections (4(3.33%)), 3(2.5%) and 6(5.00%) and than the treated birds 1(0.80%), 0(0.00%) and 2(1.67%) and is inconsistent to 4(20.0%) reported by Abu-Akkada and Awad (2015).This may be due to the differences in the number of the study birds, management operation and genetic background.

The study also showed that infection of broilers with various developmental stages of *Eimeria tenella* elicited both cellular and humoral immune responses. Homologous and heterologous challenges of the birds with the various developmental stages of the parasite at primary, secondary and tertiary infection levels stimulated the secretion and proliferation of lymphocytes and antibodies. This is in concordance with the findings of Chapman *et al*. (2005) who reported that the primary infection with *Eimeria tenella* oocyst induced complete protection against homologous challenges.The findings from the study revealed the immunogenicity of the developmental stages of the *Eimeria tenella* and this is consistent with the reports of Molloy *et al*. (2008) and Chow *et al.* (2011) who stated that the surface antigens (SAGs) of the different *Eimeria tenella* stages were capable of initiating humoral and cytokines responses in birds. The study reported a strong immune responses (humoral and cell mediated) in broilers infected with sporulated oocyst (sporozoite) and merozoites. This is similar to finding of Rose and. Hesketh (1987), Jenkins *et al*. (1991), Lillehoj (1998), Kiani and Farhang (2008) and Molloy *et al*. (2008). The strong immune response can be attributed to the relative invasive features of the parasite in nature due to proteins released from micronemes which are important for host binding and invasion. The rhoptry proteins secreted during invasion to form the parasitephorous value within which the parasite resides. Also, the possession of

glicosulphosphatidylinositol (G.P.I) –linked surface antigens (SAGs) may mediate binding to the host (Taberes *et al*., 2004). However, this study is inconsistent with work of Onaga and Nakamiura (2003).

The current study also observed a relationship between different developmental stages of the parasite and immune responses. The invasive stages (sporulated oocyst-sporozoites and merozoites) of the parasite produces high number of oocysts, antibodies and CD_4 T-lymphocytes in feaces, sera and blood of the experimental birds, respectively. The cytokines generally reported in this study which include IFN- γ, IL-1, IL-2, IL-4, IL-6, TNF and TGF confirmed the findings of Oldham (2009) and Gadde *et al.* (2011).

Serum antibody levels increased rapidly on day 10 in the broilers at primary and secondary infections and day 5 at tertiary infection. This varied from the reports Bumstead *et al*. (1995) who recorded a peak of humoral immune response between day 14 and 21 post coccidial infection in birds. This may be due to differences in the immunogenic potential of the isolate, age, environment and genetic background of the birds. Antibodies remain significantly high in broilers infected with sporulated oocyst (sporozoite) and merozoite at the end of each infection period, suggesting that the level of antibodies appears to be related to the severity of the developmental stage of the parasite. This is in concordance with the reports of Constantinoiu *et al*. (2007) who reported high antibodies levels persistence in commercial flock after natural exposure to *Eimeria* or following infection with live vaccine. The present study revealed that there was no significant difference in the antibody titre values in the treated and non treated broilers. This is consistent with the results of Kiani and Farhang (2008), but is inconsistent with the reports of Kurkure *et al*. (2006) who stated that chicks treated with coxynil showed higher antibody titre values than

those maintained on feed without coxynil. There are still debates on antibodies inducing protective immunity. Dalloul and Lillehoj, (2003) stated that antibodies play a minor role as cell mediated immunity (CMI). Gilbert *et al.* (1998), Talebi and Mulcahy (1995), reported that the levels of serum antibodies following infection do not correlate with protection or oocyst output and antibody levels in chickens. This variation may be due to the age, dose and strain of the parasite as well as the genetic background of the broilers. However, the study agrees with the findings of Rose (1987) who showed that antibody could have deleterious effects , including agglutination, lysis, neutralization of infectivity and morphological changes on various developmental stages of *Eimeria* if they come in close contact with the parasite. The first subunit vaccine ($CoxAbic^R$) is based on transfer of protective antibodies from immunized hens to embryo (Belli *et al*., 2004), indicating that antibodies do play an important role in immunity.

The present study revealed an increase in the number of CD_4 cells at day 10 post primary infection in both treated and non treated broilers as shown by Lillehoj (1998). Our study also recorded high numbers of CD_4 cells after secondary infection of the birds at day 10 as against day 6 recorded by Lillehoj, (1998). This difference may be due to differences in the age of the broilers, the strain of the parasite used or the genetic background of the birds (Bucy *et al*.,1998). The present study showed the expression of CD_4 cells subset by blood lymphocytes of broilers infected with the various developmental stages of *Eimeria tenella*. This may indicate the induction of adaptive immune response to the infection (Gadde *et al*., 2013). The CD_4 cell count expresses the numerical reactions of the broilers to oral administration of the different developmental stages of *Eimeria tenella*, revealing the stimulation of the immune system. This agrees with the reports of Hong *et al*. (2006) and

Lemus *et al*. (2010).The study also revealed that the number of CD_4 cells were significantly higher in the treated, than non-treated broilers. This is consistent to the finding of Hong *et al*, (2006) who reported higher number of CD_4 lymphocytes in infected birds treated than the non-treated ones.

The present study also demonstrated that the CD_4 lymphocytes count increased at the different periods of infection with the various developmental stages of the parasite in both the treated and non- treated broilers. This is similar to the study of Bassey *et al.* (1996) who observed that the CD_4 changes follow the phases of the parasite cycle for the *Eimeria* species considered. There was also no significant difference in the CD_4 cells count in both the treated and non-treated birds at secondary and tertiary infections. This is in accordance with the reports of Bassey *et al.* (1996) in infections of birds with *Eimeria tenella*. In summary, this work add to our understanding of the ability of the various developmental stages of *Eimeria tenella* to induce immune responses in the chicken.

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

The following can be concluded from the results obtained:

1. The study successfully harvested all the *Eimeria tenella* developmental stages (unsporulated oocyst, sporulated oocyst, schizonts, merozoites and gametocytes) which are rarely available in literature
2. The study demonstrated varying levels of oocyst outputs (groups II – 8.36 x 10^6 – 7.84 x 10^6 – 5.10 x 10^6; III - - 6.58 x 10^6 – 4.83 x 10^6; IV – 7.18 x 10^6 – 7.00 x 10^6 – 3.83 x 10^6; V – 6.59 x 10^6 – 5.87 x 10^6 – 4.20 x 10^6) and gross caecal lesions in broilers infected associated with the different developmental stages of the parasite.
4. An immune response against caecal coccidiosis could be established by immunization with *Eimeria tenella*-specific sporulat ed oocyst (sporozoites) and merozoites as well as other stages in birds of less than four weeks old
5. The sporozoites and merozoites showed strong infectivity and elicited stronger immune responses(antibody titre values in infected broilers with sporulated oocysts 0.265 ± 0.010, 0.282 ± 0.005; 0.305 ± 0.002, 0.316 ± 0.010 and 0.252 ± 0.002, 0.281 ± 0.010) and merozoites (0.177 ± 0.001, 0.186 ± 0.003; 0.135± 0.010, 0.141 ± 0.002 and 0.069 ± 0.004, 0.139 ± 0.005) in infected birds at primary-secondary-tertiary infections, indicating that they might be potential vaccines candidates against avian coccidiosis.
6. The prominent cytokines detected in the infected broilers were IFN- γ , IL-2, IL-4, IL-6, TNF and TGF, while the immunoglobulins wesre IgG or IgY.

7. Circulating CD_4 lymphocytes subset count increased ((groups I – 198.0 x 10^3 µl, 165.3 x 10^3 µl; 200.0 x 10^3 µl, 156 x 10^3 µl and 196.7 x 10^3 µl, 173.3 x 10^3 µl ; II – 199.0 x 10^3 µl, 186.0 x 10^3 µl ; 197.0 x 10^3 µl, 192.7 x 10^3 µl and 200.0 x 10^3 µl, 194 x 10^3 µl; III – 198 x 10^3 µl, 153.3 x 10^3 µl ; 200.0 x 10^3 µ,l 160.0 x 10^3 µl and 188.7 x 10^3 µl, 166.7 x 10^3 µl ; IV – 193.3 x 10^3 µl, 183 x 10^3 µl; 198.7 x 10^3 µl, 183.3 x 10^3 µl and 190 x 10^3 µl , 188.0 x 10^3 µl ; V – 200.0 x 1 0^3 µl, 198.0 x 10^3 µl ; 187.3 x 10^3 µl , 174 x 10^3 µl and 188.7 x 10^3 µl, 175.3 x 10^3 µl respectively) with the duration of infection.

6.2 Recommendations

Based on the present study, the following recommendations are made:

1. Further studies be done by interested reseachers on the immunogenicity of the sporulated oocysts and merozoites (first, second and third generations) of *Eimeria tenella* in broilers.
2. New molecular techniques be developed or invented by researchers to manipulate the genomes of the host-parasite stages interaction as well as identify the antigens involved in protection.

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APPENDICES

Appendix I : Mean O.D values of ELISA at 492nM of sera in infected broiler chickens with different developmental stages of *Eimeria tenella* at primary infection treated and non treated

Groups	Treatment	Day2	Day4	Day6	Day8	Day10
I (USO)	NT	0.028±0.002	0.030±0.001	0.054±0.002	0.062±0.010	0.067±0.003
	T	0.020±0.001	0.173±0.004	0.020±0.004	0.058±0.003	0.029±0.010
II (SO)	NT	0.171±0.013	0.173±0.004	0.178±0.010	0.181±0.002	0.282±0.005
	T	0.157±0.003	0.161±0.010	0.153±0.003	0.173±0.004	0.265±0.010
III (SCZ)	NT	0.044±0.001	0.048±0.010	0.055±0.033	0.060±0.010	0.081±0.002
	T	0.037±0.003	0.043±0.001	0.050±0.010	0.057±0.003	0.069±0.004
VI (MRZ)	NT	0.160±0.002	0.164±0.001	0.169±0.010	0.171±0.003	0.186±0.003
	T	0.158±0.003	0.149±0.010	0.160±0.002	0.169±0.010	0.177±0.001
V (GMT)	NT	0.041±0.00	0.043±0.002	0.047±0.004	0.050±0.003	0.056±0.005
	T	0.038±0.010	0.041±0.00	0.044±0.001	0.048±0.010	0.053±0.002
VI (CONTROL)		0	0	0	0	0

Appendix II : Mean O.D values of ELISA at 492nM of sera in infected broiler chickens with different developmental stages of *Eimeria tenella* at secondary infection treated and non treated

Groups	Treatment	Day2	Day4	Day6	Day8	Day10
I (USO)	NT	0.031±0.001	0.036±0.005	0.037±0.003	0.042±0.010	0.050±0.003
	T	0.029±0.010	0.030±0.002	0.033±0.004	0.039±0.001	0.041±0.002
II (SO)	NT	0.287±0.003	0.292±0.016	0.295±0.010	0.298±0.001	0.316±0.010
	T	0.279±0.001	0.286±0.003	0.290±0.001	0.289±0.004	0.305±0.002
III (SCZ)	NT	0.073±0.004	0.065±0.010	0.058±0.001	0.061±0.002	0.066±0.005
	T	0.069±0.003	0.058±0.004	0.060±0.010	0.065±0.001	0.064±0.002
VI (MRZ)	NT	0.191±0.003	0.187±0.001	0.153±0.010	0.147±0.005	0.141±0.002
	T	0.185±0.001	0.190±0.010	0.134±0.002	0.138±0.003	0.046±0.002
V (GMT)	NT	0.057±0.004	0.061±0.001	0.044±0.013	0.042±0.003	0.046±0.002
	T	0.048±0.002	0.059±0.003	0.040±0.003	0.038±0.010	0.042±0.004
VI (CONTROL)		0	0	0	0	0

Appendix III : Mean O.D values of ELISA at 492nM of sera in infected broiler chickens with different developmental stages of *Eimeria tenella* at tertiary infection treated and non treated

Groups	Treatment	Day5	Day7	Day11	Day14	Day17	Day 20	Day 24
I (USO)	NT	0.150±0.003	0.156±0.005	0.171±0.003	0.166±0.002	0.169±0.010	0145±0.006	0.167±0.005
	T	0.141±0.010	0.150±0.003	0.169±0.001	0.157±0.004	0.149±0.004	0.141±0.003	0.160±0.002
II (SO)	NT	0.281±0.010	0.233±0.004	0.199±0.001	0.186±0.003	0.179±0.001	0.161±0.005	0.154±0.010
	T	0.252±0.010	0.210±0.010	0.187±0.003	0.178±0.002	0.172±0.004	0.153±0.002	0.147±0.004
III (SCZ)	NT	0.056±0.003	0.058±0.004	0.062±0.001	0.068±0.003	0.073±0.010	0.081±0.001	0.091±0.004
	T	0.252±0.005	0.050±0.003	0.053±0.010	0.061±0.010	0.068±0.003	0.078±0.003	0.091±0.001
VI (MRZ)	NT	0.139±0.005	0.127±0.013	0.062±0.001	0.097±0.001	0.071±0.03	0.067±0.004	0.064±0.010
	T	0.069±0.004	0.123±0.005	0.053±0.006	0.093±0.003	0.065±0.005	0.054±0.010	0.051±0.006
V (GMT)	NT	0.071±0.010	0.089±0.002	0.091±0.004	0.107±0.002	0.112±0.002	0.120±0.010	0.123±0.002
	T	0.069±0.001	0.074±0.010	0.087±0.001	0.103±0.010	0.108±0.005	0.101±0/003	0.110±0.002
VI (CONTROL)		0	0	0	0	0	0	0