Comparative Analysis of Egg Adapted Vaccines and Salinomycin against Coccidiosis in Chicks

A thesis submitted in partial fulfilment of the requirements for the degree of

MASTER OF PHILOSOPHY

IN

PARASITOLOGY

By

Wajiha

Department of Animal Sciences

Faculty of Biological Sciences

Quaid-i-Azam University

Islamabad

2016

DECLARATION

I hereby declare that the work accomplished in this thesis is the result of my own research carried out in Parasitology lab, Department of Animal Sciences, Quaid-i-Azam University, Islamabad.

This thesis has neither published previously nor does it contain any material from the Published resources that can be considered as the violation of the international copy right law.

I also declare that I am aware of the term ‘copy right’ and ‘plagiarism’. I will be responsible for the consequences of any violation to these rules (if any) found in this thesis. This thesis has been checked for plagiarism.

Wajiha

CERTIFICATE

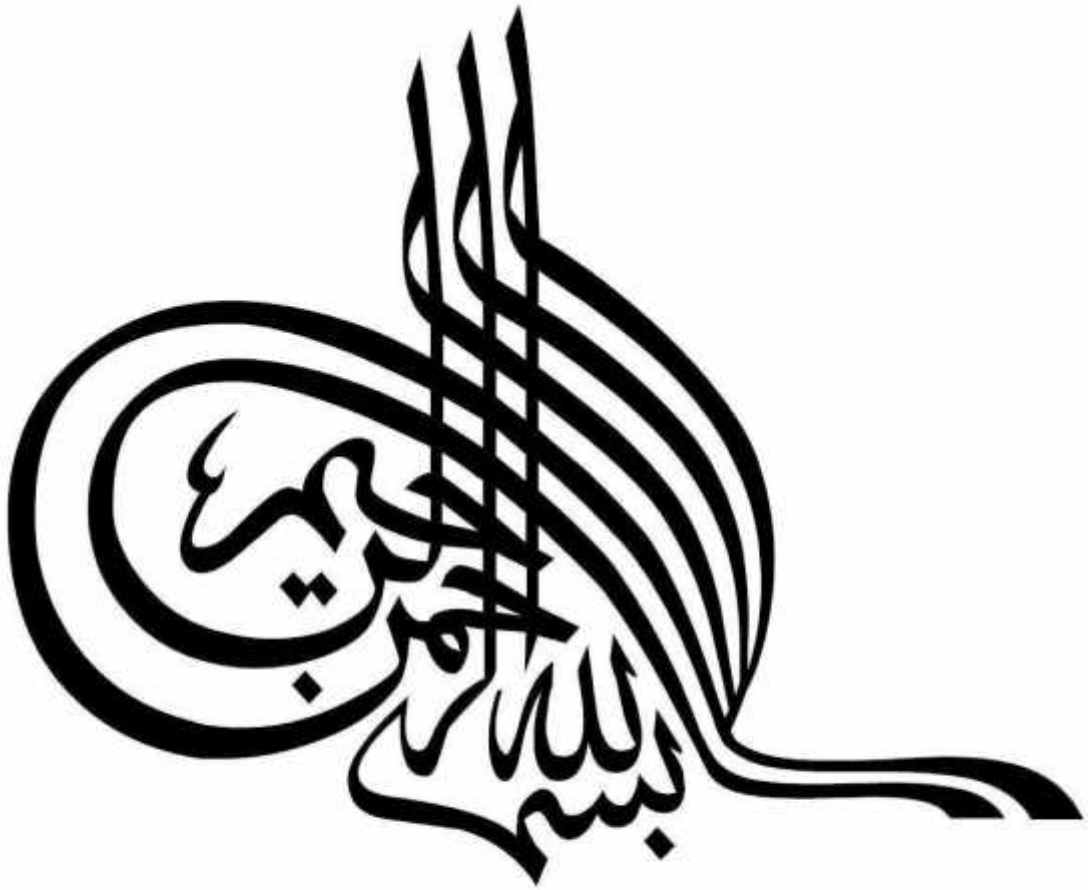
This dissertation "Comparative Analysis of Egg Adapted Vaccines and Salinomycin against Coccidiosis in Chicks" submitted by Ms. Wajiha is accepted in its present form by the Department of Animal Sciences, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad as satisfying the thesis requirements for the degree of Masters of Philosophy in Parasitology.

Internal Examiner: _____

(Supervisor)

External Examiner: _____

Chairperson: _____



In the name of Allah,
the Most Beneficent,
the Most Merciful

DEDICATION

I dedicate this humble effort

To my beloved

Parents,

Whose affection, love, encouragement and prays of

Days & night make me able to get such success and

Honour.

LIST OF CONTENTS

S. No	Title	Page No.
I	List of Tables	**viii**
ii	List of Figures	**ix**
iii	Acknowledgment	**x**
iv	Abstract	**xi**
1	Introduction	**1**
2	Materials and Methods	**7**
3	Results	**14**
4	Discussion	**28**
5	Conclusion	**32**
6	References	**33**

LIST OF TABLES

S. No	List	Page No.
3.1	Showing the comparative effect of vaccines and drug on body weight gain (mean ± SD) of birds infected with *Eimeria spp*.	14
3.2	Showing the comparative effect of vaccines and drug on feed consumption (mean ± SD) of birds infected with *Eimeria spp.*	16
3.3	Showing the comparative effect of vaccines and drug on feed conversion ratio (mean± SD) of birds infected with *Eimeria spp.*	17
3.4	Showing oocyst shedding ($\times 10^3$/gram feces) in birds infected with *Eimeria spp.*	18
3.5	Showing comparative bloody diarrhea, lesion score, survival and mortality rate of *Eimeria spp* Infected birds of experimental groups.	20
3.6	Degree of hemagglutination versus time at 37°C in serum of all experimental groups collected on 5th day of vaccination.	21
3.7	Degree of hemagglutination versus time at 37°C in serum of all experimental groups collected on 15th day of vaccination.	21
3.8	Showing mean ± SD of serum biochemical values of different samples from different experimental groups.	24

LIST OF FIGURES

S. No	List	Page No.
2.1	Showing (A). Unsporulated oocysts, (B). Sporulated oocysts.	7
2.2	Showing completely developed giemsa stained gametocytes separated from chorio allantoic fluid of in ovo inoculated egg with *Eimeria spp* sporulated oocysts.	9
2.3	Showing (A and B). Caeca filled with blood and (C). Showing normal caecum.	11
3.1	Showing body weight gain (mean ± SD) of all experimental groups in experimental period (1^{st} to 3^{rd} week of infection).	15
3.2	Showing feed consumption (mean ± SD) of all experimental groups from 1^{st} to 3^{rd} week of infection.	16
3.3	Showing feed conversion ratio (mean ± SD) of all experimental groups in experimental period (1^{st} to 3^{rd} week of infection).	17
3.4	Showing oocysts count per gram of feces (mean ± SD) of infected groups from 6^{th} to10^{th} day post infection.	18
3.5	Showing degree of hemagglutinition in vaccinated birds.	20
3.6	Showing uric acid and creatinine level in birds infected with *Eimeria spp.*	25
3.7	Showing ALP and AST level in birds infected with *Eimeria spp*.	25
3.8	Showing ALT and albumin level in birds infected with *Eimeria spp*.	25
3.9	Showing comparative histopathological observations of birds caecum of experimental groups after infection.	26
3.10	Showing comparative histopathological observations of liver of experimental groups after infection.	27

ACKNOWLEDGEMENT

*My first thanks goes to "**Almighty Allah** "the most benevolent; Whose blessings and exaltations flourished my thoughts and enabled me to improve my knowledge up to this stage. I thank **ALLAH** that in spite of all problems, which I encountered during M.Phil. enabled me to complete this dissertation. I offer my humble and sincerest words of thanks to our beloved Holy prophet, **Hazrat Muhammad (PBUH)** whose teachings guided us towards light of knowledge.*

*My heartiest compliments goes to my supervisor, **Dr. Naveeda Akhtar Qureshi,** Assistant Professor, Department of Animal Sciences, Quaid-i-Azam University Islamabad for her guidance, co-operation and knowledgeable help. I am gratefully indebted to Prof **Dr. Sarwat Jahan**, Chairperson, Department of Animal Sciences, Quaid-i-Azam University, Islamabad.*

*I also pay regards to **Dr. Farhan Afzal,** (Poultry Research Institute, Rawalpindi) for their technical help and sample collection. I am indeed humbly grateful to my friend **Rabia Afridi** for her cooperation, valuable suggestions, guidance, enlightening and delightful company during my research work.*

*I am grateful to all my lab fellows and friends **Rabia Afridi**, **Irfan Hussain**, **Amina Malik, Farah Obaid, Aalmgir, Asma Ashraf, Attiya Iqbal, Huma Fatima**, **Fehmi, Nargis Shaheen**, **Muhammad Afzal, Qurat-ul-Ain, Amina Arshad, Farzana, Qasim Afridi, Shabir, Gulshan, Nayab Khan** for their endless cooperation, valuable suggestions and most affectionate behavior. I am obliged to **Naeem Masih** and **Sami Ullah** for their help and support and motivation during my research.*

*I can never forget the prayers and untiring efforts of my parents. They encouraged me always and sacrificed their comfort for my brilliant future. I am greatly obliged to my beloved brother **Maaz Sani** and sisters **Tahira Gul** and **Hafsa Gul** for their prayers and encouragement during the tenure of the research work.*

Wajiha

Abstract

Coccidiosis is one of the most economically devastating disease caused by intracellular protozoan parasite of genus *Eimeria.* It is an important intestinal infection associated with metabolic and structural changes in the intestinal mucosa of the host. Since last few decades anticoccidial drugs are being used but due to abundant use of drugs, resistant *Eimeria* strains developed. In the present study three types of eggs adapted vaccines namely; egg adapted gametocytes, sonicated gametocytes and formalin inactivated gametocytes has been developed for controlling coccidiosis. Comparative efficacy of salinomycin and egg adapted vaccines against coccidiosis was investigated. Total of ninety, day old chicks (birds) were divided into six groups (A-F). On 5th day of age three groups were vaccinated orally with 0.2ml vaccines to its corresponding group. On 21st day of age all groups except control were given infection orally with dose of 10,000 oocyst/bird through dropper. On 5th day post inoculation 60mg/kg salinomycin was given to infected unvaccinated medicated group to check the comparative effectiveness of vaccines and drug. Body weight gain, feed consumption, feed conversion ratio were investigated till end of experiment. While bloody diarrhea, oocysts excretion were investigated at the first and second week of post infection. Blood samples were also collected from each group on 5^{th,} and 15th day of vaccination as well as on 7th day of infection. For histopathological examination tissue samples from intestine and caeca were taken on day 7th post inoculation from all groups. Gametocytes vaccinated and salinomycin treated group had significantly higher (P=0.002) body weight gain, feed consumption when compared with formalin and sonicated vaccinated and infected non-medicated unvaccinated groups respectively. Gametocytes vaccinated group showed feed conversion ratios similar with salinomycin medicated group showing significant association of (P=0.001). Mild bloody diarrhea and lowest oocyst count was shown by gametocyte vaccinated and salinomycin treated groups. Gametocytes vaccinated and salinomycin medicated group showed survival rate of 100% similar to negative control. Blood samples collected after infection were analyzed for biochemical tests. The results showed significant decrease in albumin concentration and increase level of uric acid, ALP, AST, ALT and creatinine in infected non medicated unvaccinated, formalin inactivated gametocytes and sonicated gametocytes vaccinated groups. Histopathological examination showed *Eimeria* induced lesion were mild in

gametocytes vaccinated, medicated and control group. While in non-immunized infected group the lesions were severe. On blood collected at day 5^{th} and 15^{th} post vaccination from vaccinated groups, indirect hemagglutination test were applied for detection of antibodies. Results of IHA test showed that in gametocytes vaccinated group blood hemagglutination is maximum as compared to remaining two vaccinated groups.

INTRODUCTION

Poultry is one of the most important industry worldwide, growing rapidly since last few decades (Mwale and Masika, 2011). The demand for poultry products has been increased with increasing world population (Windhorst, 2006). Globally 3.5 billion birds are reared annually (Zhang *et al*., 2013). One-third of meat produced and consumed worldwide is poultry meat as it is a good source of animal protein (meat and egg) (Scanes, 2007). The need and demands of poultry products are much more as compared to its production (Yegani, 2009).

According to Food and Agriculture Organization 2007, about 20 billion poultry industries exists worldwide, while 75% of this is in developing countries (Mwale and Masika, 2011). Rural areas contributes 98.5-99.2% of the poultry products with annual output of 72,300 and 78,000 metric tons of total meat and eggs respectively (Alemu *et al*., 2012). In Pakistan poultry meat contributes 24.8% of total meat, providing employment and financial gain for 1.5 million people (Bachaya *et al.,* 2012). However poultry industry is being affected by many parasites creating complication and have inhibitory effects on its growth parameters (Ruff, 1999; Bera *et al.,* 2010). Single celled intracellular protozoan parasite of genus *Eimeria* frequently affects poultry industry causing avian coccidiosis resulting in high rate mortality (Waldenstedt *et al*., 1998). Causative agent of coccidiosis is *Eimeria spp* (kingdom Protista, class Sporozoa, subclass Coccidia, order Eucoccidia, family Eimeridae and genus Eimeria) (Lilic *et al.,* 2009). Approximately 1700 *Eimeria spp* are yet described and are significant threat to different animals especially poultry causing severe clinical disease and economic loss (Yang *et al*., 2015). Among these 1700 species nine species of poultry are most common and pathogenic i.e. *E. tenella, E. brunetti, E. necatrix, E. praecox, E. mitis, E. acervulina, E. maxima, E. hagani* and *E. mivati* (Gazyagci *et al.,* 2015).

The *Eimeria spp* attacks intestinal tract causing massive destruction of the epithelial cells resulting in bloody diarrhea, reduced weight gain, temporary reduction in egg production and mortality (Masood *et al*., 2013). Worldwide the incidence of coccidiosis in poultry range from 5 to 70% (Usman *et al.,* 2011) whereas in developing

countries it is estimated between 20 to 40% (Kinung *et al*.,2004). Poultry coccidiosis, one of the most devastating disease is causing global economic loss of US$3 billion annually (Dalloul and Lillehoj, 2005; Morris and Gasser, 2006). In United Kingdom and China economic loss due to coccidiosis are more than US$70 and US$73 million annually due to costly drugs, predisposition to secondary disease and mortality (Williams, 1999; Zhang *et al*., 2013). Economic significance of coccidiosis can be gained by calculating the amount spent annually on medication. The most recent estimate indicates that in the United Kingdom, approximately 6.4 million GBP are spent annually on anticoccidial drugs (Chapman, 2009).

Life cycle of *Eimeria spp* is complex, single host and complete in two phases i.e. sexual and asexual phases. Birds acquire infection by ingesting infective oocysts (eggs) from litter, contaminated feed and water. Ingested oocysts are crushed in the gizzard releasing sporocysts. The action of trypsin and bile in the duodenum releases sporozoites, which invade the mucosa to become trophozoites. During a preclinical phase in asexual stage of development, the parasites undergo a rapid multiple fission (schizogony) resulting in formation of schizonts and hundreds of daughter cells called merozoites. Mucosal cells of intestinal tract are invaded by merozoites where they multiply resulting in formation of male and female gametocytes (Allen and Fetterer, 2002). In sexual phase microgametes and macrogametes develops into male and female gametocytes respectively and fused to form zygote which matures into oocyst. The infected birds excrete oocysts in their feces and are a source of infection for other birds (Mcdougald, 1998; Quiroz - castaneda *et al*., 2015).

Eimeria spp multiplying in the intestinal tract causes tissue damage exposing the birds to bacterial infections. Damage to mucosal lining results in blood loss and can interfere with the food digestion, nutrient absorption (Gupta, 2009). Each *Eimeria spp* shows different signs of infection. *E. tenella* and *E. necatrix* causes significant blood loss. Disruption of the absorptive surfaces of the gut, and the excretion of large amounts of mucus and fluid are the signs shown by other *Eimeria spp* (Mcdougald, 1998).

Pathogenicity due to *Eimeria* depends on many factors such as parasite *spp*, viability, infectivity, virulence, host age, immunological competence as well as

prevailing environmental conditions and management practices. It is reported that highly pathogenic species of *Eimeria* are *E. brunette, E. necatrix, E. maxima,* and *E. tenella* causing death due to severe mucosal erosion resulting in hemorrhagic enteritis, while pathogenicity cause by *E. acervulina*, *E. mivati* and *E. mitis* is of low range and *E. praecox* and *E. hagani* is lesser pathogenic as compare with other species (Bachaya *et al.,* 2012). All *Eimera spp* vary in their localization in the gut by attacking a particular site in the intestinal tract of birds and in their ability to cause the relative incidence of disease and ultimately death (Haug *et al*., 2007; Morris *et al*., 2007). For example, the upper portion of the small intestine is damaged by *E. acervulina* and entire small intestine is effected by *E. maxima* and caecum is distressed by *E. tenella* (Al-Quraishy *et al.,* 2009; Usman *et al.,* 2011). The reproductive potential also varies greatly. The lesser pathogenic *spp* such as *E. acervulina* and *E. mitis* reproduce more abundantly than the more pathogenic *spp* such as *E. tenella* and *E. necatrix* (Mcdougald, 1998). Pathogenicity also depends on host age as mortality in young birds is higher than old birds due to weak and undeveloped immunity because birds between 3 and 18 weeks are affected by most of *Eimeria Spp* (Gupta, 2009; Bera *et al*., 2010; Bachaya *et al.,* 2012).

Main cause of coccidial infection in birds is contamination with oocyst in early stages of life (Khan *et al*., 2011). Higher prevalence of infection during rainy season of monsoon observed, Indicating that warmth temperature and humid condition favors the oocyts sporulation and subsequent transmission (Amin *et al.,* 2014). Birds infested by *Eimeria spp* shows both clinical and sub clinical signs. Clinical signs include malabsorption, profuse watery to bloody diarrhea due to penetration of the intestinal wall by parasite and ultimately death. In mild infection birds show sub clinical signs like weakness and dehydration due to low feed and water intake, poor nutrient absorption, decreased capability of feed conversion, loss of skin pigment, and in layers egg production temporarily reduced (Bera *et al.,* 2010).

Several methods are being used for specific diagnosis of avian coccidiosis which includes clinical features, morphological and pathological aspects such as detecting oocysts excreted from infected birds, morphology of different stages of parasite in fecal material, gut pathology, location and extent of lesions determined during necroscopy,

examination of mucosal scarping and pre-patent period (Schnitzler *et al.,* 1998; Carvalho *et al*., 2011; Home *et al.,* 2015).

Direct diagnosis of coccidiosis is done by oocyst examination in fecal smear, the most common method. Fresh fecal samples should be collected for microscopic examination of oocyst (Mcdougald, 1998; Dezfoulian *et al.*, 2010). Oocysts per gram of feces can be counted by McMaster technique (Haug *et al*., 2008). Identification of different species on morphology of oocysts is very challenging and requires expertise (Gussem, 2006). It is suggested that lesion scoring is an interpretation based on macroscopic visible lesions caused by *Eimeria spp* usually following a scoring system from zero to four (Johnson and Reid 1970). However Amare *et al.,* (2012) reported that histopathological examination of intestine and liver tissues is also a reliable method for diagnosis. All *Eimeria spp* produce lesion of gross appearance at a specific location in the gastrointestinal tract; *E. tenella* produces bloody lesions in caeca while *E. necatrix* and *E. maxima* reproduces in small intestine but are distinguished on the basis of difference in oocysts size (Morris and Gasser, 2006). *E. brunetti* and *E. acervulina* are easily identified due to production of gross lesion (Allen and Fetterer, 2002). These methods are not only time consuming, but also unreliable due overlapping and similar morphological characteristics which creates problems in precise diagnosis of different species in a single host (Morris *et al*., 2007; Lee *et al.*, 2011). Molecular methods for differentiation of different species have been developed due to emerging knowledge of *Emeria spp* at gene level. The practical application of these molecular techniques in the field of coccidiosis have been limited (Haug *et al*., 2008). Recently, diagnosis of different *Eimeria spp* is by Polymerase Chain Reaction (PCR) based amplification of DNA (Patra *et al*., 2010).

Currently chemoprophylaxis and vaccination are used for controlling coccidiosis. Chemoprophylaxis named as anticoccidials are mostly given in feed (Chapman, 2005). The use of anticoccidial feed additives during past 50 years has played a major role in growth of poultry industry and has facilitated increased availability of high quality, affordable poultry products to the consumers (Tewari and Maharana, 2011).

Coccidiostats were discovered in 1950's and in 1964 these coccidiostats were termed as chemotherapeutic (Markiewicz *et al*., 2012). Now a days there are many substances having coccidiostatic properties (1) Synthetic coccidiostats (2) Natural coccidiostats i.e. Chemical compounds of natural origin. Synthetic coccidiostats were recently introduced and differ in their mode of action against biological activity, metabolism and chemical structure of parasite. These drugs include Robenidine, Decoquinate, Diclazuril, Halofuginone and nicarbazin (Tewari and Maharana, 2011). Natural coccidiostats kills parasite by interfering with ion transport of sodium and potassium and disrupting osmotic balance. Host cell manage the interference of these important ions, but not by parasites resulting in their death. These include narasin, monensin, lasalosid, maduramycin, salinomycin and semduramicin (Allen and Fetterer, 2002; Gupta, 2009; Markiewicz *et al*., 2012).

A couple of chemicals marketed today for control of coccidiosis are amprolium, nicarbazin, robenidin, diclazuril, zoalene, decoquinate, halofuginone. The fact that they are still being marketed is a demonstration of their value to the poultry industry and thus an indication of the more limited potential for resistance buildup compared to ones which disappeared (Gussem, 2006). The use of anticoccidial drugs has developed resistance against drugs e.g. salinomycin, monesine, narasin etc (Peek and Landman, 2003; Abbas *et al.*, 2008; Masood *et al*., 2013). Thus there is a need for novel approaches and alternative control (Dalloul and lillehoj, 2005). Over the past several decades, efforts are being made for development of vaccines (Gupta, 2009). Vaccines cost more than anticoccidials but due to continual use of chemoprophylaxis, vaccines are the major alternative to chemotherapy for coccidiosis control. (Allen and Fetterer, 2002).

Currently Paracox (consisting precocious strains of all seven *Eimeria spp*) and Livacox (consisting precocious and egg passaged lines) Vaccines are available for controlling coccidiosis in breeder and layer flocks (Shirley *et al*, 1995). Coccivac and Immucox each are composed of several virulent species (Sharman *et al.,* 2010). An advantage of attenuated vaccines low reproductive potentials, thus avoiding crowding in the specific mucosal areas of infection and resulting in the development of optimal immunity with minimal tissue damage (Allen and Fetterer, 2002).

In ovo vaccination is another type of vaccines in which live coccidian are injected in egg for the control of coccidiosis (Weber *et al.*, 2004). It is speculated that antibodies raised against surface gametocyte antigens of *Eimeria spp* could inhibit the growth, development, and fertilization of gametes and thereby stop transmission of the disease (Hafeez *et al*, 2007). Recent studies demonstrated the feasibility of immunizing birds against infection with *E. tenella* through in ovo injection of oocysts, sporocysts, or sporozoites into 9 day old chick embryos (Weber and Evans, 2003).

The present work is planned to develop egg propagated gametocytes vaccines. Sporozoites of *Eimeria spp* inoculated into the allantois cavity of ten day birds embryos. The sporozoites in egg completes their life cycle in eight to nine days at 37 °C and 70% humidity. The development of the parasite within the embryos is systematically observed, allowing guidelines to be set regarding the appropriate times at which different developmental stages of the parasite may be sampled for vaccination (Jiang *et al.*, 2012).

OBJECTIVES

1. Comparative evaluation of anticoccidial strategies i.e. Egg adapted vaccines and anticoccidial drug salinomycin.

2. Preparation of different types of vaccines from locally developed *Eimeria spp* gametocytes.

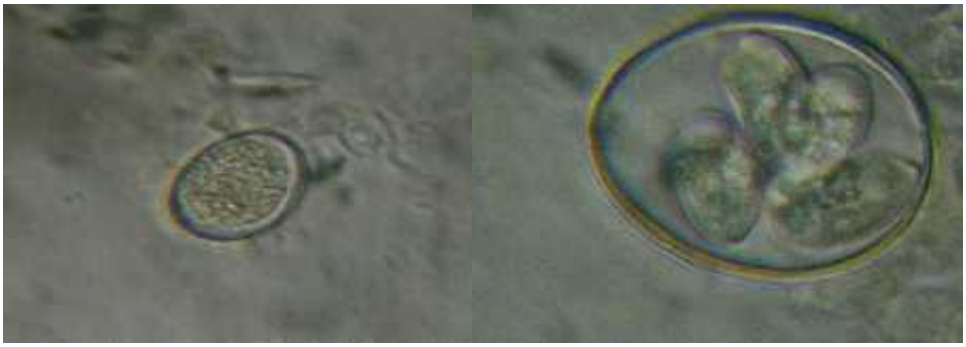
MATERIALS AND METHODS

2.1 Sample collection and examination

Coccidiosis suspected birds (chicks) guts were collected from poultry research institute (PRI) Rawalpindi in 16 to 25ml vials containing potassium dichromate ($K_2Cr_2O_7$, 2.5%) and sample in 1:5 respectively. The guts were opened by giving longitudinal incision. Contents of the caeca were examined by direct microscopic examination using low and high magnification. Positive samples were separated for sporulation of oocysts (Long *et al.,* 1976).

2.2 Sporulation and preservation of oocysts

For sporulation of oocysts, positive guts were kept in petri dishes containing 2.5% potassium dichromate in 1:5 ratio at room temperature for 7-10 days (Amer *et al*., 2010; Sharma *et al*., 2013). Sporulated samples were homogenized in phosphate buffered distilled water having pH 8 for 2-3 minutes. Trypsin was added to homogenized solution, incubated at 41°C for 30 minutes and was strain through muslin cloth. In 15ml centrifuge tubes filtrate was centrifuged at 1500 rpm for 10 minutes, supernatant was discarded. The remaining solution (pallet) containing sporulated oocysts was given 2 to 3 washings and placed in a sterilized eppendorf tubes at 4° C in refrigerator till further use (Amer *et al*., 2010).



(A) (B)

Figure 2.1: Showing (A). Unsporulated oocysts, (B). Sporulated oocysts.

2.3 Embryos in ovo inoculation

18 hen eggs (10 day old) were maintained at 39°C and 70% humidity in an incubator. Candling was performed daily for embryo status. Eggs (12 day age) from incubator were placed on an egg plate with air space upside and desired area was swabbed with 70% alcohol. At opposite side of embryo head a small hole was made in egg shell with sterile needle. 0.1ml suspension containing sporulated oocysts along with penicillin was inoculated into the allantois cavity of each embryo using 1ml syringe by inserting about ¾ of needle. Two embryos were kept as control by inoculation of phosphate buffer saline water. Molten wax was used for sealing hole in egg shell. Eggs were again incubated for 5 to 7 days at standard temperature and humidity (Jiang *et al*., 2012; Brauer and Chen, 2015).

2.4 Development and collection of gametocytes

The inoculated eggs with sporulated oocysts passed through all developmental stages of life cycle on chorio allantois membrane. After 168 hours post inoculation, both mature macro and microgametes were observed microscopically (Jiang *et al*., 2012). On day 7th post infection, chorio allantoic fluid was collected from each embryo. Egg shell was disinfected using 70% alcohol. The shell above air space was cracked and removed using sterile forceps and a pair of scissors. The chorio allantois membrane was ruptured and fluid was aspirated by sterile syringe. The fluid from each egg was collected in separate sterile eppendorf tubes and centrifuged at 800 rpm for 20 minutes at 4°C to remove RBC's. Embryo debris were separated from gametocytes by treating with 1% trypsin at 3°C for 2 hours and washed with sterile normal saline solution by centrifugation for 5 minutes at 1500 rpm. Purified gametocytes were stored at 4°C in minimum volume of normal saline for further use (Hafeez *et al*., 2006; Hafeez *et al*., 2007). The existence of gametocytes was tested by spreading on glass slide, air dried, stained with eosin and observed under microscope (Jiang *et al*., 2012).

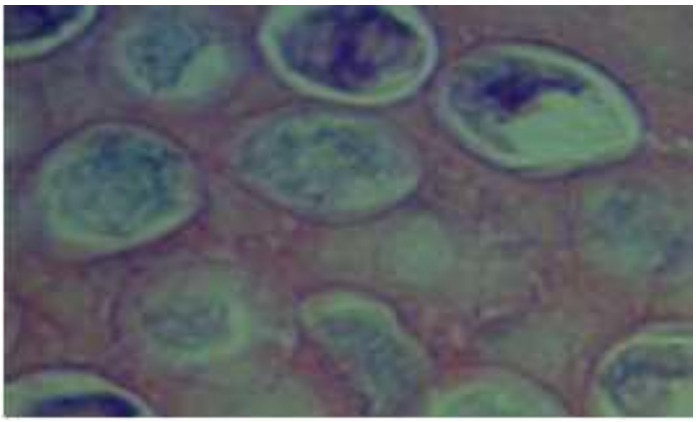


Figure 2.2: Showing completely developed giemsa stained gametocytes separated from chorio allantoic fluid of in ovo inoculated egg with *Eimeria spp* sporulated oocysts.

2.5 Vaccines preparation

Vaccines of 3 different types were prepared from egg adapted *Eimeria spp.*

Vaccine I: Gametocytes

Vaccine II: Formalin inactivated gametocytes

Vaccine III: Formalin inactivated sonicated gametocytes

Gametocytes were sonicated using ultrasonic homogenizer for 5 minutes and centrifuge at 250 rpm for 15 minutes. Collected supernatant was inactivated with 3% formalin (Ayaz *et al.*, 2008; Anwar *et al.*, 2008).

2.6 Experimental design

Day old ninety birds were procured from local hatchery of Rawalpindi. Birds were reared in coccidian free environment at animal house. Room and cages were washed with detergents and antiseptic before placing birds. Coccidiostat free feed was given to all birds except control group till end of experiment (Christaki *et al.*, 2004). On 5^{th} day of age, birds were divided into 6 groups (A-F). Each group was in triplicate with 5 birds (n=5) per cage. At 5^{th} day groups (A-C) were orally given 0.2 ml vaccine (I-III) (gametocytes, formalin inactivated and formalin inactivated sonicated gametocytes) respectively. Group 'D' was infected and medicated, while Group 'E' was infected unvaccinated, non-medicated. Group 'F' was given no infection and kept as negative control. The experiment was conducted up to 42 days. The experimental groups except negative control were challenged orally with *Eimeria spp* 6,000 to 7,000 oocysts/bird on the 15^{th} day of post

vaccination (Hafeez *et al.*, 2006). Birds started shedding oocysts after five days of infection and medicated group D was given salinomycin in feed at 60mg/kg body weight (Lee *et al*., 2013).

The comparative effect vaccines and drug (salinomycin) was evaluated on basis of parameters such as, clinical signs and symptoms, mean weight gain, feed consumption, feed conversion ratio, oocysts per gram of feces, diarrhea, lesion score, survival and mortality rate.

2.7 Mean weight gain determination

All chicks present in different cages were weight on 21st day of age i.e., on day of infection and this weight was considered as the initial weight. Chicks were again weighted on 4th, 5th and 6th week of age prior to feeding using weighing balance.

Mean weight gain (MWG) was calculated using following formula:

MWG = (Mean final weight of live chicks in a cage) - [(Mean initial weight of all chicks in that cage)+ (weight of dead chicks)] (Naidoo *et al.*, 2008; Lee *et al.*, 2013).

2.8 Feed consumption

Throughout the experimental period standard quantity of feed was given to each group and next morning prior to feeding the left over feed of each group on was weighed back to determine the feed consumption of each group.

2.9 Determination of feed conversion ratio

Feed conversion ratio (FCR) is gram of feed consumed by birds to produce one gram of their body weight. FCR was calculated from 1st to 3rd week post infection. Feed conversion ratio for individual birds cannot be determined because birds were feed as a group.

The feed conversion ratio (FCR) was calculated using the formula (Naidoo *et al*., 2008).

FCR = Total feed consumed per group (g) / (weight gain of live birds + weight gain of dead birds).

2.10 Oocysts per gram of feces (OPG)

Fecal samples from each cage were collected from 5th to 10th day post infection for microscopic examination of oocysts (Christaki *et al*., 2004). McMaster's technique was used for counting the oocysts. 3 grams of fecal sample were mixed with 42ml of tap

water in a beaker. In case if pellets were present they were broken and suspension was kept for 12 to14 hours. Suspension was then passed through two folds muslin cloth.

For calculating the oocysts count per gram of fecal sample following formula was used,

$$X/0.15\times 45 \times 10 \times 1/3 = X1000$$

Where X= Average oocyst count in 1 chamber, 0.15= Volume under ruled area of McMaster slide, 45= Total volume of suspension, 10= 1/10 dilution factor, 1/3=Correction factor.

2.11 Bloody diarrhea

Bloody diarrhea was observed from 4^{th} to 7^{th} day of infection. The extent of bloody diarrhea was assigned as one of five levels, where the zero or (-) level is the normal status, 1 or (+) corresponds to less than 33%, 2 or (++) to corresponds 26–50%, 3 or (+++) to 51–75%, while 4 or (++++) to over 75% bloody feces in total feces (Christaki *et al*., 2004).

2.12 Lesion score

Birds were euthanized on 7^{th} and 14^{th} day of infection from each group. After post mortem intestinal tract of each bird was examined and lesion score of 0-4 was assigned to each group (Johnson and Reid, 1970).

A B C

Figure 2.3: Showing (A and B). Caeca filled with blood and (C). Showing normal caecum.

2.13 Survival percentage

Survival percentage was calculated as follows (Christaki *et al*., 2004; Du and Hu, 2004).

$$\text{Survival percentage} = \frac{\text{No of live birds at the end of experiment}}{\text{Total no of live birds}} \times 100$$

2.14 Mortality percentage

Mortality percentage was calculated using the formula (Christaki *et al*., 2004; Naidoo *et al*., 2008).

$$\text{Mortality percentage} = \frac{\text{Total no of dead birds in the cage}}{\text{Initial no of birds in the cage}} \times 100$$

Clinical signs, symptoms, feed conversion ratios, oocysts per gram of feces counting, weight and mortality record in each group was maintained during experimental period (Ryley *et al.*, 1976; Hafeez *et al.*, 2006).

2.15 Biochemical analysis

Blood samples were collected from wing vein of birds from all groups on 5^{th} and 15^{th} day of post vaccination and 7^{th} day of the post infection through 3ml syringes in separate vacutainers with anticoagulant. Then centrifuged at 750 rpm for 15 minutes at 4°C. The serum was stored in eppendorf tubes at – 20°C until to be analyzed. The serum determination of albumin, uric acid, creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) activities by using commercial kits and biochemical analyzer (Hafeez *et al.*, 2006; Hashemnia *et al*., 2014).

2.16 Indirect Hemagglutination test

The serum and RBC's (collected on 5^{th} and 15^{th} day of the post vaccination) were used for detection of antibodies titer and developed immunity in vaccinated birds by indirect hemagglutination test. Erythrocytes of human blood group 'O' were suspended in sterile phosphate buffer saline and washed three times to remove plasma or other constituents of blood other than erythrocytes by centrifugation at 1500 rpm for 5 minutes. The erythrocytes (0.5ml) suspended in 10ml phosphate buffer saline (PBS) was

mixed with 10ml of 3 different vaccines i.e. gametocytes, formalin inactivated and formalin inactivated sonicated gametocytes vaccines (antigens) in three sterile test tubes and incubated at 37°C for different time durations from 20 to 100 minutes. After incubation, sensitized erythrocytes containing solution was washed twice with PBS by centrifugation. 96 wells of micro titration plate were filled with 100µl of PBS. First row was filled with 100µl of serum sample. 50µl of sensitized RBC's of human blood group "O" were added to each well except last one to which washed RBC's were added as control. Serum and RBC's were gently mixed by tapping. The plates were incubated at 25°C for 25 minutes. Degree of hem agglutination in each well was recorded (Sultana *et al.*, 2014).

2.17 Histopathology

Tissues samples of liver and caecum were taken from all groups on 7th day of infection after post mortem. Tissues were fixed in 10% formalin for further processing. Samples were washed with different concentrations (70, 80, 90, 95 and 100%) of ethanol and cleared in xylene for 60-90 minutes. The specimens were embedded in paraffin wax for 120 minutes and ribbons were made after sectioned with 7µ thickness by microtome machine. A thin layer of albumin was smeared on slides by placing on slides warmer. Ribbons on albumin coated slides were placed in xylene, 100, 90, 70% ethanol for 10 minutes. Slides were gently washed with water for 2 minutes. Then stained with hematoxyline for 10 seconds and again washed with running water for 5 minutes. Eosin stain was applied for 1 minute and de-stained with 95% ethanol. Slides were coated with four drops/slide of permount and observed under light microscope (Kawazoe *et al*., 2005; Kadhim, 2014).

2.18 Statistical analysis

The data of present experiment was statistically analyzed using software. Results were presented as mean ± standard deviation. Differences between means were determined using one way analysis of variance (ANOVA) and the mean values were compared by Tukey test. The differences among group means were considered significant at $P<0.05$.

RESULTS

All experimental groups except negative control showed clinical signs such as in appetence, weakness, rough feathers, followed by bloody diarrhea at 6th day and being severe at 7th to 9th day of infection. The same clinical signs with low severity were observed in gametocyte vaccinated and medicated groups.

3.1 Comparative effect of vaccines and drug on growth of birds after infection

From 1st to 3rd week post infection unexpected changes in mean body weight gain of *Eimeria* infected birds were observed. Mean body weight gain of uninfected, non-medicated and unvaccinated control group was higher than all vaccinated, medicated and control groups with mean value of 430±4.04 gm. Gametocytes vaccinated group showed maximum weight gain of 417±5.29 gm which was approximately similar to mean weight gain (412±8.50 gm) of infected medicated control group, showing significance difference of (P=0.004). Mean weight gain of these two groups were statistically in nearest range to negative control group. Lowest weight gain (278±5.13 gm) was recorded in infected non-medicated un vaccinated control group. While group B and C treated with formalin inactivated gametocytes and sonicated gametocytes showed statistically similar weight gain of 385±7.63 and 386±7.57 gm respectively (Table and Figure 3.1).

Table 3.1: Showing the comparative effect of vaccines and drug on body weight gain (mean ± SD) of birds infected with *Eimeria spp***.**

Time (week)	**Mean body weight gain (gm) from 1st to 3rd week post infection**					
	Group A	Group B	Group C	Group D	Group E	Group F
1st wk	226±11.84^{b}	195±13.05^{cd}	184±7.81^{d}	223±13.79^{bc}	149±10.59^{e}	309±3.00^{a}
2nd wk	344±11.15^{b}	301±13.00^{c}	297±14.36^{c}	339±7.81^{b}	231±4.04^{d}	391±3.21^{a}
3rd wk	417±5.29^{ab}	385±7.63^{c}	386±7.57^{c}	412±8.50^{b}	278±5.13^{d}	430±4.04^{a}

Values having different superscripts in same row are significantly different from one another by Tukey test (P=0.004).

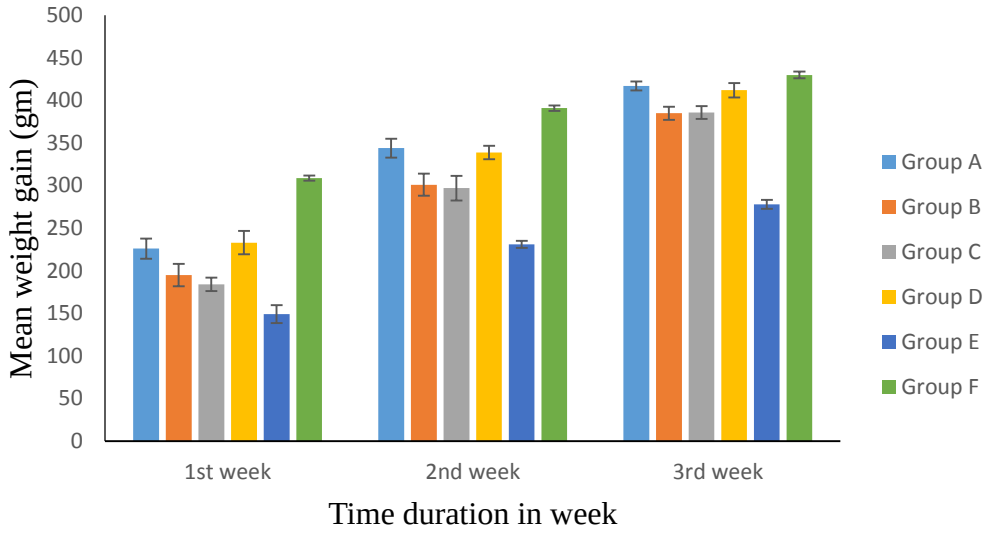


Figure 3.1: Showing body weight gain (mean ± SD) of all experimental groups in experimental period (1st to 3rd week of infection).

3.2. Comparative analysis of mean feed consumption of vaccinated and medicated groups with control groups after infection

Among vaccinated groups maximum feed of 707±2.64 gm was consumed by group 'A' (gametocytes vaccinated) which was approximately similar to feed consumed by uninfected non-medicated unvaccinated and salinomycin medicated control groups with mean values of 753±5.03 gm 712±3.60 gm respectively showing significance association of P=0.003. While vaccinated groups 'B' and 'C' showed mean feed consumption of 699±1.73 gm and 696±5.56 gm respectively, similar to 681±8.02 gm mean feed consumption of group E (Table and Figure 3.2).

Table 3.2: Showing the comparative effect of vaccines and drug on feed consumption (mean ± SD) of birds infected with *Eimeria spp***.**

Time (week)	Feed consumed (gm) by birds during 1-3 weeks post infection.					
	Group A	Group B	Group C	Group D	Group E	Group F
1st wk	450±10.01^{ab}	431±15.27^{bc}	429±12.09^{bc}	447±7.63^{ab}	409±3.15^{c}	470±2.08^{a}
2nd wk	589±8.14^{ab}	581±7.02^{b}	581±7.09^{b}	584±7.23^{b}	532±7.76^{c}	603±3.51^{a}
3rd wk	707±2.64^{bc}	699±1.73^{c}	696±5.56^{c}	712±3.60^{b}	681±8.02^{d}	753±5.03^{a}

Values having different superscripts in same row are significantly different from one another by Tukey test (P=0.003).

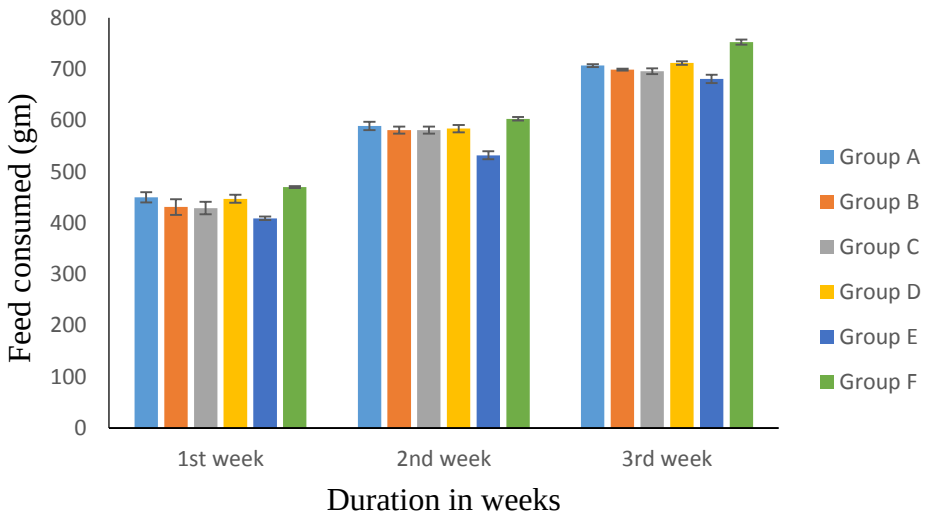


Figure 3.2: Showing feed consumption (mean ± SD) of all experimental groups from 1st to 3rd week of infection.

3.3 Comparative analysis of different groups on basis of feed conversion ratio

Groups A, D, F showed lowest feed conversion ratio of 1.69±0.02, 1.72±0.04 and 1.74±0.00 respectively, showing significance value of (P=0.002). While groups B, C and E due to severe infection and less feed consumption showed higher values of feed conversion ratio i.e., 1.81±0.03, 1.79±0.04 and 2.44±0.04 respectively (Table and Figure 3.3).

Table 3.3: Showing the comparative effect of vaccines and drug on feed conversion ratio (mean ± SD) of birds infected with *Eimeria spp.*

Time (week)	Feed conversion ratio (FCR) of birds during 1st to 3rd week post infection.					
	Group A	Group B	Group C	Group D	Group E	Group F
1st wk	1.99±0.14^{b}	2.21±0.12^{b}	2.27±0.06^{b}	2.00±0.09^{b}	2.74±0.18^{a}	1.51±0.01^{c}
2nd wk	1.70±0.07^{cd}	1.92±0.06^{b}	1.95±0.11^{b}	171±0.05^{c}	2.29±0.02^{a}	1.53±0.01^{d}
3rd wk	1.69±0.02^{c}	1.81±0.03^{b}	1.79±0.04^{b}	1.72±0.04^{bc}	2.44±0.04^{a}	1.74±0.00^{bc}

Values having different superscripts in same row are significantly different from one another by Tukey test (P=0.002).

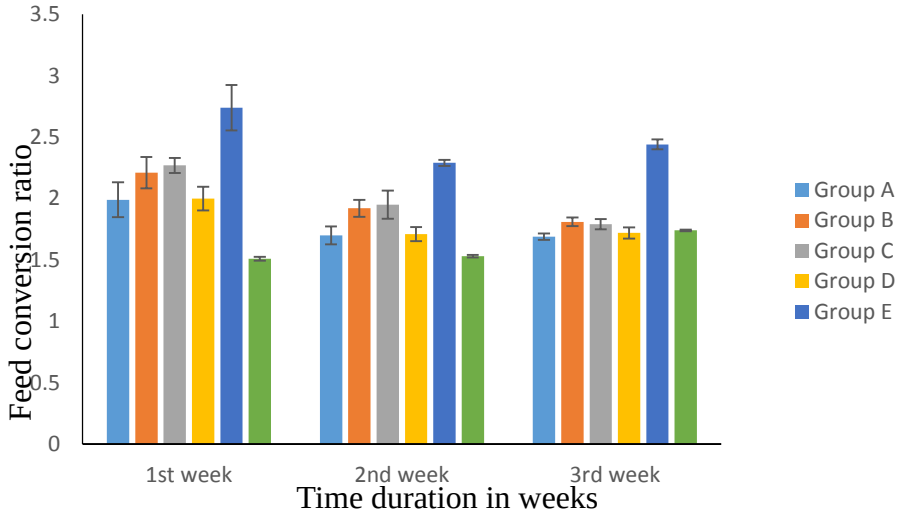


Figure 3.3: Showing feed conversion ratio (mean ± SD) of all experimental groups in experimental period (1st to 3rd week of infection).

3.4 Comparative effect of vaccines and drug on oocyst count per gram of feces of birds infected with *Eimeria spp.*

The effect of vaccines and drug on oocyst count per gram of feces of infected birds were calculated during first two weeks of infection. Highest oocysts count were recorded on 7th and 8th day post inoculation in all infected groups except negative control group which showed no oocsyts shedding. Gradual decrease in oocysts shedding was observed after 8th day of infection in all groups. Highest oocysts count of 36.31±3.18 was shown by

infected, unvaccinated, non-medicated control group. Salinomycin treated group showed total oocysts count of 23.31±2.30 which was similar to oocysts count (22.31±2.05) of gametocytes vaccinated group, with P value equal to 0.006**.** Vaccinated groups B and C (formalin inactivated and formalin inactivated sonicated gametocytes) showed oocysts count of 25.98±2.07 and 27.31±2.24 respectively (Table and Figure 3.4).

Table 3.4: Showing oocyst shedding ($\times 10^3$/gram feces) in birds infected with *Eimeria spp.*

Time Duration	**Means ± SD Oocyst per gram of feces ($\times 10^3$)**					
	Group A	Group B	Group C	Group D	Group E	Group F
6th day	2.66±0.57^{b}	3.66±0.57^{b}	3.33±0.57^{b}	2.33±1.52^{b}	4±1.00^{a}	00±00^{c}
7th day	5.33±0.57^{b}	5.66±0.57^{b}	5.66±0.57^{b}	4.33±1.52^{b}	7.66±0.57^{a}	00±00^{c}
8th day	7.33±0.57^{b}	8.33±1.73^{b}	8.66±2.00^{b}	7.66±1.52^{b}	9.00±1.00^{a}	00±00^{c}
9th day	4.66±0.57^{ab}	5.33±1.52^{bc}	6.33±1.00^{c}	4.00±1.00^{ab}	6.33±0.57^{a}	00±00^{d}
10th day	2.33±0.57^{b}	3.00±1.00^{b}	3.33±1.00^{ab}	2.66±1.00^{b}	4.33±0.57^{a}	00±00^{c}
Total	22.31±2.05	25.98±2.07	27.31±2.24	23.31±2.30	36.31±3.18	00±00

Values having different superscripts in same row are significantly different from one another by Tukey test (P=0.006).

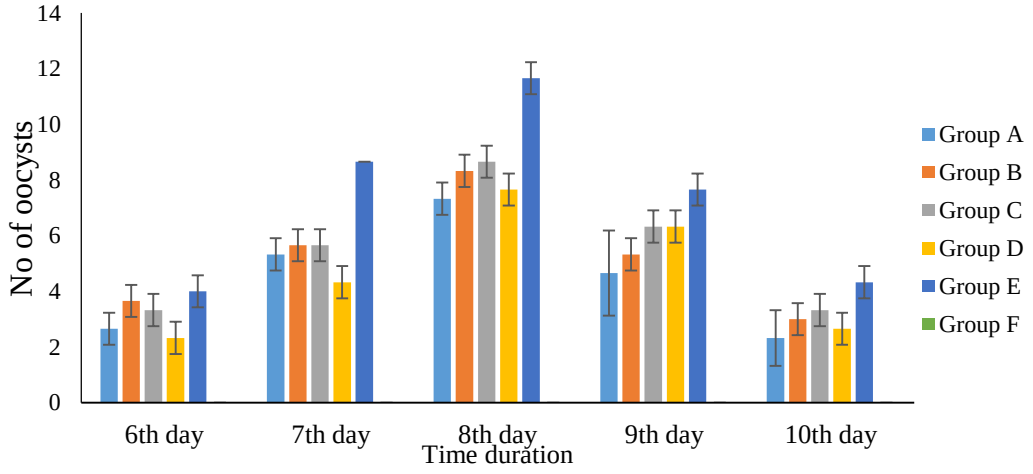


Figure 3.4: Showing oocysts count per gram of feces (mean ± SD) of infected groups from 6th to 10th day post infection.

3.5 Comparative effect of vaccines and drug on bloody diarrhea, lesion score, mortality rate and survival rate of experimental groups

On 4th day post infection birds in all groups except uninfected control group showed bloody diarrhea. In infected, non-medicated, unvaccinated group severe bloody diarrhea was observed from 5th to 7thday post infection. In salinomycin medicated and gametocytes vaccinated groups mild diarrhea was observed which disappeared after 5th day. While remaining two vaccinated groups (group B, C) showed mild to moderate diarrhea on 4th, 5th and 6th day post inoculation. On 7th and 8th day of infection bloody diarrhea disappeared in all groups except infected, non-medicated, unvaccinated control group (Table 3.5).

Maximum lesion score of grade +4 was observed in infected, non-medicated, unvaccinated group. Lesion score of +2 grade was examined in formalin treated gametocytes vaccinated group 'B' and grade +1 lesion score was observed in sonicated gametocyte vaccinated group 'C'. salinomycin medicated and negative control group showed lesion score of zero grade (Table 3.5).

Whereas no mortality occurred in gametocyte vaccinated, salinomycin medicated and non-infected groups respectively. Maximum mortality (50%) was observed in infected non-medicated control group, followed by formalin inactivated and sonicated gametocytes vaccinated groups with mortality rate of 20, 40% respectively (Table 3.5).

Maximum survival rate of 100% was shown by gametocytes vaccinated, salinomycin medicated and non-infected groups. Vaccinated group B (gametocytes inactivated with formalin) showed 80% survival rate followed by group C (sonicated gametocytes) and infected non-medicated group E with survival rate of 60 and 50% respectively (Table 3.5).

Table 3.5: Showing comparative bloody diarrhea, lesion score, survival and mortality rate of *Eimeria spp* infected birds of experimental groups.

Groups	Bloody diarrhea (Day)					Lesion score	Mortality rate (%)	Survival rate (%)
	3rd	4th	5th	6th	7th			
A	_	+	+	_	_	+0	0	100
B	_	+	++	+	_	+2	20	80
C	_	+	++	+	_	+1	40	60
D	_	+	+	_	_	+0	0	100
E	_	++	+++	++	+	+4	50	50
F	_	_		_	_	+0	0	100

3.6 Indirect Hemagglutination test results

In IHA test extent of hemagglutination was clearly observed in serum samples collected on 5th and 15th day post vaccination in vaccinated groups. More agglutination in a sample means maximum antibodies titer present in sample. No agglutination occurred at room temperature. Maximum agglutination was observed after plates were kept in incubator at 37°C for 40 minutes. Highest antibodies titer was present in group A (gametocytes vaccinated) after 15th day post vaccination. Group B and C showed moderate level of agglutination (antibodies titer) (Table 3.6, 3.7 and Figure 3.5).

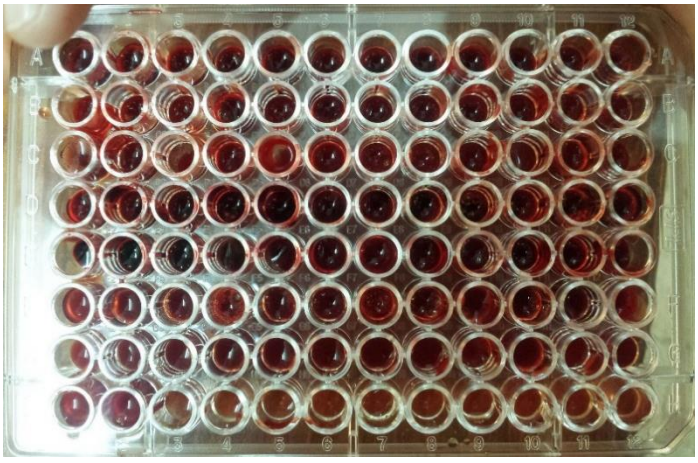


Figure 3.5: Showing degree of hemaggluitination in vaccinated birds.

Table 3.6: Degree of hemagglutination versus time at 37°C in serum of all experimental groups collected on 5th day of vaccination.

Time (min)	Group A	Group B	Group C	Group D	Group E	Group F
20	–	–	–	–	–	–
40	++	–	–	–	–	–
60	+++	+	+	–	–	–
80	L	++	+	–	–	–
100	L	L	++	–	–	–

+ = Low reaction, ++ = Moderate reaction, +++ = Strong reaction, L = Lysis of red blood cells, – = No reaction.

Table 3.7: Degree of hemagglutination versus time at 37°C in serum of all experimental groups collected on 15thday of vaccination.

Time (min)	Group A	Group B	Group C	Group D	Group E	Group F
20	–	–	–	–	–	–
40	+++	–	–	–	–	–
60	L	++	+	–	–	–
80	L	+++	+	++	–	–
100	L	L	++	++	+	–

+ = Low reaction, ++ = Moderate reaction, +++ = Strong reaction, L = Lysis of red blood cells, – = No reaction.

3.7 Serum biochemical analysis of different experimental groups

A significant increase in uric acid concentration of 8.69±0.01 mg/dl was observed in infected, non-medicated, unvaccinated group when compared with negative control group with uric acid concentration of 6.22±0.05 mg/dl. Salinomycin medicated group showed uric acid concentration of 6.33±0.08 mg/dl which was statistically similar to negative control group and gametocytes vaccinated group (6.06±0.04) showing significance association of (P=0.002). Higher uric acid concentration of 7.57±0.41 mg/dl

was observed in vaccinated group C which was more than vaccinated groups A and B i.e., 6.06±0.04 and 6.97±0.04 mg/dl respectively (Table 3.8 and Figure 3.6).

Infected non-medicated control group showed significant increase in creatinine concentration with value of 1.46±0.08 mg/dl. Concentration of creatinine decreased to 0.87±0.09 mg/dl in negative control group which was approximately similar to gametocyte vaccinated and salinomycin medicated groups with concentration of 0.86±0.12 and 0.80±0.05 mg/dl respectively showing significance association of (P=0.004). While remaining vaccinated groups B and C showed concentration of 1.02±0.03 and 1.10±0.08 mg/dl respectively (Table 3.8 and Figure 3.6).

A significant decrease in alkaline phosphatase (ALP) concentration 233±24.57U/l was observed in infected, unvaccinated, non-medicated group when compared with negative control group. Similarly vaccinated groups B and C showed decreased ALP value of 268±10.05 and 256±18.44U/l respectively. Gametocytes vaccinated group showed significance association of (P=0.002) with medicated and negative control groups with ALP concentrations of 303±3.79, 292±11.12U/l and 322±13.97U/l respectively (Table 3.8 and Figure 3.7).

In negative control group aspartate aminotransferase AST concentration was 281±4.15 U/l which was approximately near to values of vaccinated group A and medicated group D with AST concentration of 266±9.53 U/l and 264±10.2 U/l respectively, thus showing significance association of P=0.003. Significant increase in AST concentration (316±9.08 U/l) was observed in infected, non-medicated, unvaccinated control group. While gradual increase in concentration of AST occurred in remaining two vaccinated group B and C i.e., 299±6.63 and 298± 5.35 U/l respectively (Table 3.8 and Figure 3.7).

Normal level of alanine amino transferase (ALT) was observed in control group F which was 5.57±0.30 U/l. Vaccinated group ‘A’ and medicated group ‘D’ showed approximately similar level of ALT i.e., 5.37±0.16 and 5.06±0.08 U/l respectively thus showing significance association of (P=0.004). Vaccinated group ‘B’ and ‘C’ showed gradual increase in ALT concentration with values of 12.58±0.24 and 11.82±1.07 U/l

respectively. Higher concentration (13.39±0.37 U/l) of ALT was shown by infected, non-medicated unvaccinated group (Table and Figure 3.8).

Pre-infection value of albumin was 1.28±0.01 gm/dl in negative control group. Albumin concentration decreased significantly to 0.69±0.07 gm/dl in infected, non-medicated unvaccinated group. While in vaccinated group 'A' and salinomycin treated group the level of albumin was observed as 1.26±0.06 and 1.22±0.03 gm/dl respectively which was similar to non-infected control group showing significance association of (P=0.005). Gradual increase in albumin level occurred in group B and C with values of 1.03± 0.04 and 1.38± 0.05 respectively (Table and Figure 3.8).

Table 3.8: Showing mean ± SD of serum biochemical values of different samples from different experimental groups.

Parameters	Biochemical values (mean ± SD)					
	Group A	**Group B**	**Group C**	**Group D**	**Group E**	**Group F**
Uric Acid	6.06 ± 0.04^{d}	6.97 ± 0.04^{c}	7.57 ± 0.41^{b}	6.33 ± 0.08^{d}	8.69 ± 0.01^{a}	6.22 ± 0.05^{d}
Creatinine	0.86 ± 0.12^{c}	1.02 ± 0.03^{bc}	1.10 ± 0.08^{b}	0.80 ± 0.05^{c}	1.46 ± 0.08^{a}	0.87 ± 0.09^{c}
ALP	303 ± 3.79^{ab}	268 ± 10.05^{bcd}	256 ± 18.44^{cd}	292 ± 11.12^{abc}	233 ± 24.57^{d}	322 ± 13.97^{a}
AST	266 ± 9.53^{c}	299 ± 6.63^{ab}	298 ± 5.35^{ab}	264 ± 10.23^{c}	316 ± 9.08^{a}	281 ± 4.15^{bc}
ALT	5.37 ± 0.16^{c}	12.58 ± 0.24^{ab}	11.82 ± 1.07^{b}	5.06 ± 0.08^{c}	13.39 ± 0.37^{a}	5.57 ± 0.30^{c}
Albumin	1.26 ± 0.00^{a}	1.03 ± 0.04^{ab}	1.38 ± 0.05^{a}	1.22 ± 0.03^{a}	0.69 ± 0.00^{b}	1.28 ± 0.01^{a}

Values having different superscripts in same row are significantly different from one another by Tukey test.

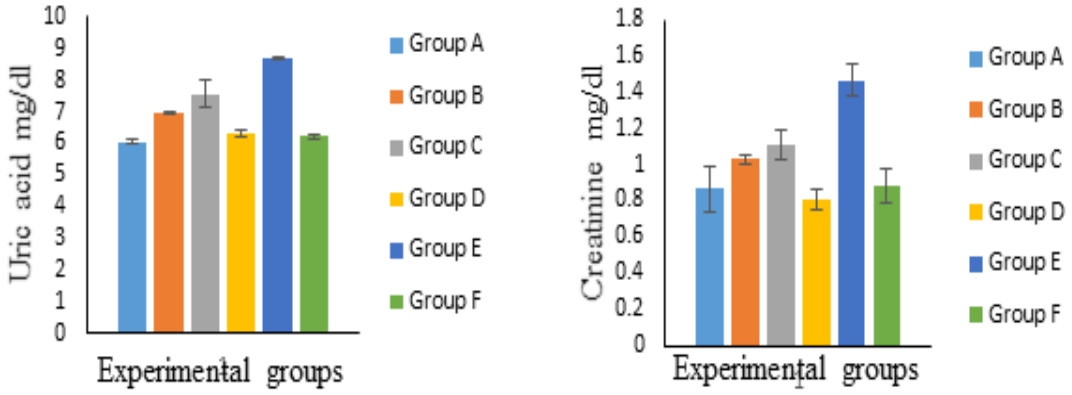


Figure 3.6: Showing uric acid and creatinine level in birds infected with *Eimeria spp.*

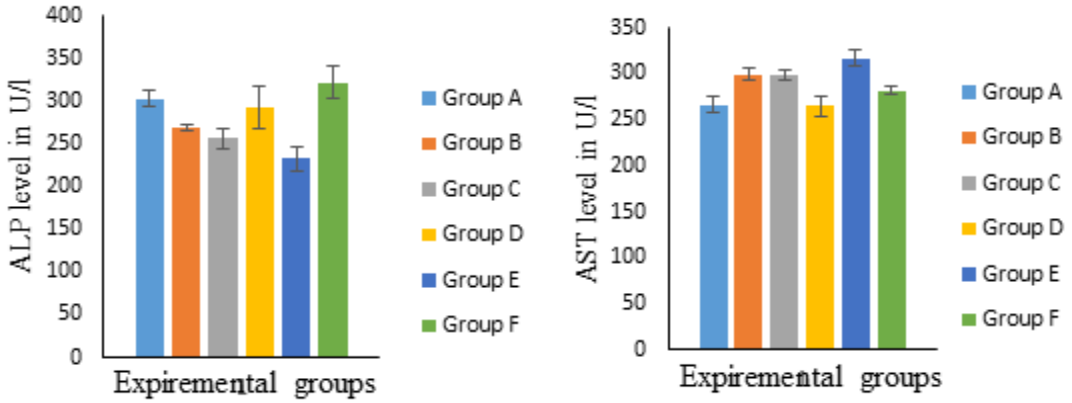


Figure 3.7: Showing ALP and AST level in birds infected with *Eimeria spp*.

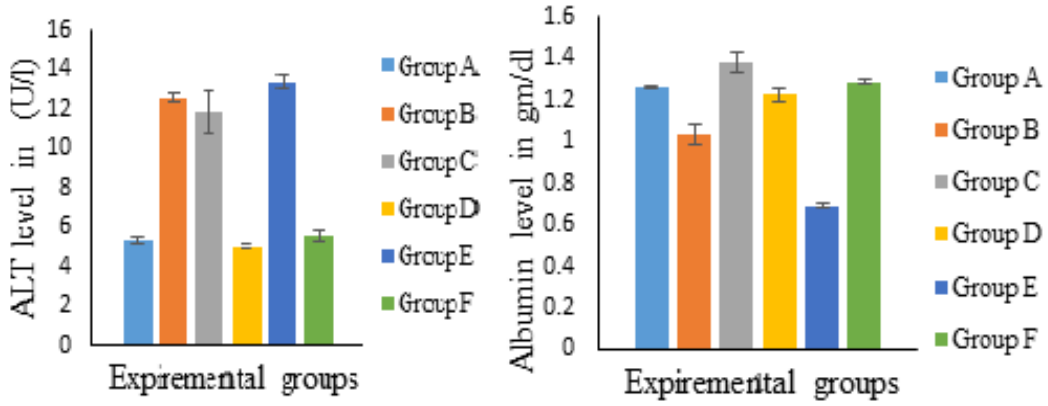


Figure 3.8: Showing ALT and albumin level in birds infected with *Eimeria spp*.

3.8 Histopathology

Infestation of *Eimeria spp* was apparent in caecum and liver. Majority of histopathological lesions were observed in these infected birds on 7th day after infection.

3.8.1: Comparative histopathological observations of chicken's caeca after infection

Microscopic examination of permanent histopathological slides showed normal caeca with no lesions in gametocytes vaccinated, salinomycin medicated and negative control groups. Histopathological findings of formalin inactivated and sonicated gametocytes vaccinated birds caecum showed presence of schizonts in intercellular spaces and congested and blocked blood vessels in mucosal layer of caecum. Infected, non-medicated, unvaccinated control group severe histopathological lesions were clearly observed with presence of schizont stages, fusion of villi and discrete hemorrhage (Figure 3.9).

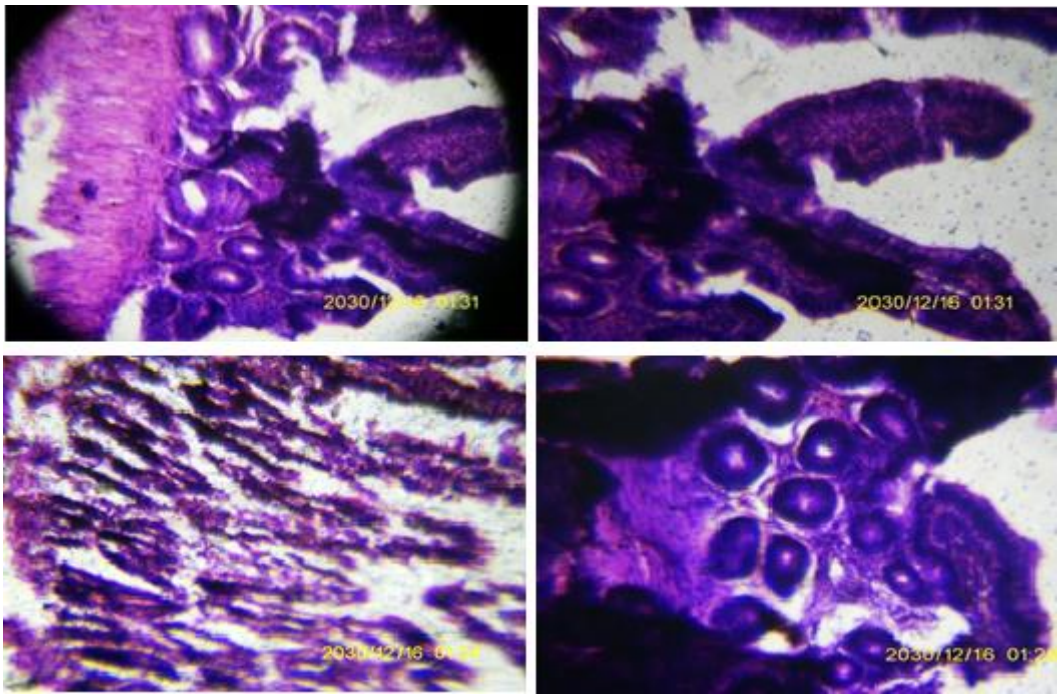


Figure 3.9: Showing comparative histopathological observations of birds caecum of experimental groups after infection.

3.8.2: Comparative histopathological observations of birds liver after infection.

Eimeria spp also affects parenchymal cells of liver. Infestation of *Eimeria* parasite produce severe histopathological lesions in liver. Negative control and gametocytes vaccinated birds showed normal histology of liver. In formalin inactivated and sonicated gametocytes vaccinated birds moderate lesions were observed in liver i.e. presence of clotted and partly clotted blood. Severe lesion were observed in liver of positive control birds (Figure 3.10).

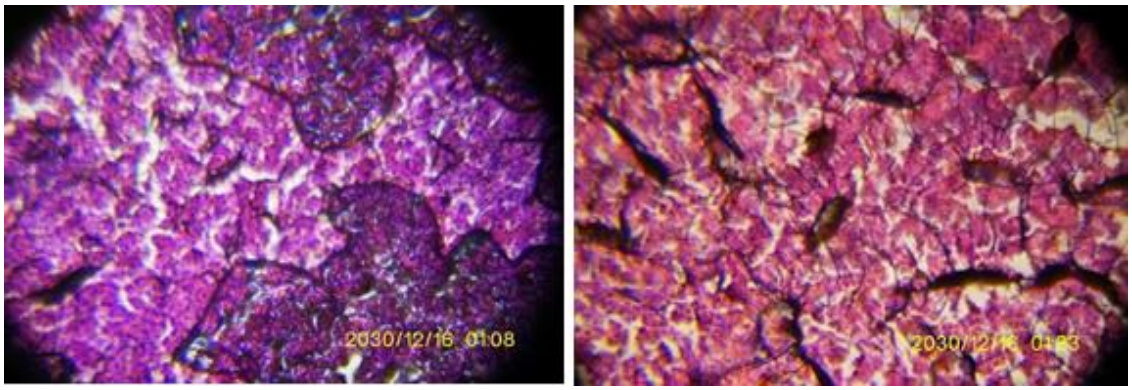


Figure 3.10: Showing comparative histopathological observations of liver of experimental groups after infection.

DISCUSSION

Coccidiosis one of the most devastating disease is caused by single cell intracellular protozoan of genus *Eimeria* (Dalloul and Lillehoj, 2005; Lilic *et al.,* 2009). Coccidiosis is a parasitic disease of intestinal tract by causing high mortality in chicks (Zhang *et al.,* 2012). *Eimeria spp* is highly immunogenic. Primary infection of coccidiosis stimulate cascade of immune reactions against reinfections (Lillehoj and Lillehoj, 2000). Different anticoccidial drugs are used as preventive strategies to control coccidiosis as it is impossible to rear large number of chicks together without anticoccidial chemotherapeutic measurements (Morris and Gasser, 2006). Due to frequent use of chemotherapeutic, drugs resistant strains have been developed (Masood *et al*., 2013). This create need for novel and alternative control strategies in form of development of vaccines (Gupta, 2009).

In present study comparative anticoccidial strategies were used for control of coccidiosis i.e., live locally developed vaccines of *Eimeria spp* and anticoccidial drug salinomycin (Weber *et al.,* 2004; Lee *et al*., 2013). Egg adapted vaccination strategy is effective for control of coccidiosis due to loss of virulence and decreased reproductive potential. Salinomycin is most widely used anticoccidial drug and *Eimeria spp* developed resistance against it (Peek and Landman, 2003). Due to development of resistance to salinomycin, continuous and excessive use of salinomycin may create some physiological abnormalities in chicks (Rizvi *et al*., 2008).

Purpose of this study was to evaluate the comparative effect of egg adapted vaccines and anticoccidial drug salinomycin. The results of different parameters in this experiment were investigated from 1st to 3rd week of infection in all groups.

Higher mean body weight gain and feed consumption was shown by gametocytes vaccinated chicks when compared with vaccinated with formalin inactivated gametocytes and formalin inactivated sonicated gametocytes. Similar results of higher weight gain was shown by work of Anwar *et al*., (2008) who reported significant increase in mean body weight gain and feed consumption in local gametocytes vaccinated chicks compared to LivaCox vaccinated chicks. Similarly Lee *et al*., (2012) reported higher weight gain and feed consumption in

vaccinated chicks when compared with medicated chicks. Likewise, Danforth (1998), Jenkins *et al*., (2010) showed higher weight gain in immunized and salinomycin medicated broilers when compared with control group. In contrast Lehman *et al.,* (2009) demonstrated that medicated broilers had greater body weights compared with coccidia-vaccinated chickens during the first 3 weeks of infection.

Results showed feed conversion ratio of gametocytes vaccinated chicks was less than medicated and others two vaccinated chicks i.e. formalin inactivated gametocytes and formalin inactivated sonicated gametocytes vaccinated chicks which was similar to results of Del Cacho *et al.,* (2012) who immuinized chicks with dendritic cell derived exosomes vaccines had reduced FCR ratio when compared with infected non immunized chicks. Likewise, Danforth, (1998), Williams and Gobbi, (2002) and Harfoush *et al.,* (2010) reported lowest FCR chicks treated with ionophores when compared with infected untreated chicks.

Gametocytes vaccinated chicks showed lowest oocysts count which was similar to findings to Lee *et al*., (2012) whose study showed lower oocysts count per gram of feces in ovo vaccinated chicks when compared with ionophore medicated chicks. Highest oocysts count was recorded in infected, unmedicated, unvaccinated control group which was similar to results of Morris *et al.,* (2007) showing highest oocysts count in positive control group. In contrast, results of Stanley *et al., (*2004) reported the presence of highest oocysts in the litter of broilers on medicated diets. Razmi and Kalideri, (2000); Sun *et al*., (2009) reported that differences in oocysts counts may be due to differential effects of anticoccidial drugs and vaccines.

Mild bloody diarrhea was observed in gametocytes vaccinated chicks when compared with positive control group. Work of Bahrami and Bahramu, (2006) also shows results of mild diarrhea in vaccinated chicks due to development of strong immunity. Similarly Akhtar *et al.,* (2001) reported that in gametocytes vaccinated chicks no diarrhea is observed, while in formalin inactivated gametocytes vaccinated chick severe diarrhea were observed.

No mortality occurred in gametocyte vaccinated, salinomycin medicated and in negative control groups. Hafeez *et al*., (2007) also reported no mortality in immunized chicks (gametocytes vaccinated) while in positive control chicks four out of five chicks died due to coccidiosis. Similarly, William 2002 reported lower mortality in vaccinated birds when compared with drug-treated birds.

Lower lesion score was observed in vaccinated group (gametocytes), medicated group and uninfected negative control group. Similarly Anwar *et al*., (2008) reported local gametocyte and LivaCox immunized chicks had low grade lesions. Danforth, 1998 also reported lower mild intestinal lesion score in vaccinated birds when compared with non-immunized control birds. Decreased damage to the caecal mucosa in immunized chicks suggests the involvement of immune effector mechanism that may have resulted in inhibition of development of early stages of the parasite's life (Anwar *et al*., 2008).

IHA antibodies titer were significantly higher in gametocytes vaccinated chicks when compared with formalin inactivated and formalin inactivated sonicated gametocytes vaccinated chicks. Anwar *et al.,* (2008) showed higher antibody titres in local gametocyte-vaccinated group as compared to LivaCox®-vaccinated chicks. Dalloul *et al.,* (2006) reported significantly higher antibodies titer in chicks vaccinated in ovo with mushroom derived lectin when compared with positive control chicks. According to Sultana *et al*., (2014) antibodies titer was higher in sonicated sporulated oocysts vaccinated than non-immunized group. On contrast, Lee *et al*., (2012) investigated highest serum anticoccidial titers in birds fed a non-medicated diet when compared with vaccinated chicks.

A significant increase in uric acid concentration were observed in infected positive control chicks when compared with non-infected negative control group. Harfoush *et al.*, (2010) and Patra *et al*., (2010) also suggested that in infected chicks due to severe infection of coccidiosis uric acid concentration were increased from normal level. Manyawe *et al*., (2010) reported that in *Eimeria spp* infected chicks increase level of uric acid is due to kidney dysfunction, metabolic acidosis as well as intravascular hemolysis.

Significant increase in creatinine concentration was observed in infected positive control chicks when compared with non-infected negative control. According to Zhang *et al*., (2012) and Harfoush *et al.*, (2010) creatinine concentration increases from normal level in infected chicks. Mathivanan and Edwin, (2012) investigate that due to liver and kidney pathology as well as due to impaired dehydration creatinine level were increase from normal level in infected chicks.

Aspartate aminotransferase (AST) and alanine amino transferase (ALT) concentration increased in infected, non-medicated, unvaccinated chicks. Similarly Harfoush *et al.*, (2010) and Hashemnia *et al*., (2014) also investigated increase concentration of (AST) and (ALT) in infected group. Vahdatpour *et al.,* (2011) reported that due to acute hepatic degenerations and damage in liver and muscle tissues caused increase in (AST) and (ALT).

Infected, unvaccinated, non-medicated control chicks showed decreased alkaline phosphatase (ALP) concentration. Similar result of decreased (ALP) concentration were found in studies by Patra *et al*., (2010) and Abd El-Maksoud *et al*., (2014). Sivaraj *et al.*, (2011) investigate that (ALP) level is decreased due to severe bone marrow damage.

Albumin concentration decreased significantly in infected, non-medicated, unvaccinated group similar with findings of Patra *et al*., (2010). Concentration of albumin decreases from normal level due to renal failure resulting in excretion of albumin outside body. Liver damage is also main reason of decreasing albumin level as it is a site for albumin synthesis (Sharman *et al.,* 2010).

In gametocytes vaccinated chicks low level of histopathological lesions were observed while severe lesions in caeca and liver were induced in infected non immunized chicks. Kadhim, (2014) also found similar results of severe intestinal, caecal and liver lesions due to congestion of blood vessels. Negative control group in present study showed normal mucosa, sub mucosa and crypt cells which is also proved by study of Dezfoulian *et al.*, (2010) and Kawazoe *et al.*, (2005).

CONCLUSION

Current investigation revealed that effect of live *Eimeria spp.* gametocytes vaccine (experimentally developed) against coccidiosis is significantly more effective than formalin inactivated and formalin inactivated sonicated gametocytes vaccines as well as from salinomycin. The salinomycin currently used to cure coccidiosis is developing not only resistance in *Eimeria spp* but its continuous use may also create some physiological complications in birds like chicks as reported by Rizvi *et al.,* 2008.

REFERENCES

Abbas, R. Z., Iqbal, Z., Sindhu, Z. D., Khan, M. N. & Arshad, M. (2008). Identification of cross-resistance and multiple resistance in *Eimeria tenella* field isolates to commonly used anticoccidials in Pakistan. *Journal of Applied Poultry Research*, 17(3): 361–368.

Abd El-maksoud, H. A., Afaf, D., Abdel-magid. & El-badry, M. A. (2014). Biochemical effect of coccidian infestation in laying hen. *Benha Veterinary Medical Journal*, 26(1): 127-133.

Akhtar, M., Hayat, C. S., Ayaz, S., Ashfaque, M., Ayaz, M. M. & Hussain, I. (2001). Development of immunity to coccidiosis in chicken administrated sonicated coccidial vaccine. *Journal of Pakistan Veterinarni,* 21: *2*

Alemu, D., Degefe, T., Ferede, S., Nzietcheung, S. & Roy, D. (2012). Overview and Background Paper on Ethiopia's Poultry Sector. Relevance for HPAI Research in Ethiopia, (1).

Allen, P. C. & Fetterer, R. H. (2002). Recent Advances in Biology and Immunobiology of *Eimeria Spp* and in Diagnosis and Control of Infection with These Coccidian Parasites of Poultry. *Clinical Microbiology Review*, 15(1): 58–65.

Allen, W. M., Berrett, S., Hein, H. & Hebert, C. N. (1973). Some physio pathological changes associated with experimental *Eimeria brunetti* infection in the chicken. *Journal of Comparative Physiology*, 83(2): 369-375.

Al-Quraishy, S., Abdel-Baki, A. S. & Dkhil, M. A. (2009). *Eimeria tenella* infection among broiler chicks Gallus domestics in Riyadh city, Saudi Arabia. *Journal of King Saud University Science*, 21(3): 191–19.

Amare, A., Worku, N. & Negussie, H. (2012). Coccidiosis prevailing in parent stocks: A comparative study between growers and adult layers in Kombolcha poultry breeding and multiplication center, Ethiopia. *Journal of Global Veterinarian*, 8(3): 285–291.

Amer, M. M., Awaad, M. H. H., El-Khateeb, R. M., Nadia, M. T. N., Abu-Elezz., Sherein-Said, A., Ghetas, M. M. & Kutkat, M. A. (2010). Isolation and Identification of Eimeria from Field Coccidiosis in Chickens. *Journal of American Science*, 6(10):1107–1114.

Amin, Y., Aslam, A., Anwar, K. & Ali, Z. (2014). Seasonal prevalence of eimeriosis in broiler chicken. *International Journal of Advance in Life Sciences*, 1(3): 160–164.

Anwar, M. I., Akhtar, M., Hussain, I., Haq, A. U., Muhammad, F., Hafeez, M. A. & Bashir, S. (2008). Field evaluation of Eimeria tenella (local isolates) gametocytes vaccine and its comparative efficacy with imported live vaccine, LivaCox. *Journal of Parasitology Research*, *104*: 135–143.

Ayaz, M. M., Akhtar, M., Hussain, I., Muhammad, F. & Haq, A. U. (2008). Immunoglobulin producing cells in chickens immunized with Eimeria tenella gametocyte antigen vaccines. *Journal of Veterinarni Medicines*, 53(4): 207–213.

Bachaya, H. A., Raza, M. A., Khan, M. N., Iqbal, Z., Abbas, R. Z., Murtaza, S. & Badar, N. (2012). Predominance and detection of different *Eimeria Spp* causing coccidiosis in layer chickens. *Journal of Animal and Plant Sciences*, 22(3): 597–600.

Bahrami, A. M. & Bahrami, A. (2006). Immune response of chicken to an experimental sonicated coccidia oocyst vaccine. *Journal Archives of Razil Institutes*, 61(1): 43–48.

Bera, A. K., Bhattacharya, D., Pan, D., Dhara, A., Kumar, S. & Das, S. K. (2010). Evaluation of Economic Losses due to Coccidiosis in Poultry Industry in India. *Agricultural Economics Research Review*, 23(1): 91–96.

Brauer, R. & Chen, P. (2015). Influenza Virus Propagation in Embryonated Chicken Eggs. *Journal of Visualized Experiments*, 19(97): 1–6.

Carvalho, F. S., Wenceslau, A. A., Teixeira, M., Carneiro, J. A. M., Melo, A. D. B. & Albuquerque, G. R. (2011). Diagnosis of *Eimeria Spp* using traditional and molecular methods in field studies. *Journal of Veterinary Parasitology*, 176 (2-3): 95–100.

Chapman, H. D. (1984). Drug resistance in avian coccidia. *Journal of Veterinary Parasitology*, 15(1): 11–27.

Chapman, H. D. (2005). Perspectives for the control of coccidiosis in poultry by chemotherapy and vaccination. *Proceedings of the IXth International Coccidiosis Conference*, 1–5.

Chapman, H. D. (2009). A landmark contribution to poultry science Prophylactic control of coccidiosis in poultry. *Journal of poultry Scienc*e, 88: 813–815.

Christaki, E., Paneri, P. F., Giannenas, I., Papazahariadou, M., Botsoglou, N. A. & Spais, A. B. (2004). Effect of a mixture of herbal extracts on broiler chickens infected with *Eimeria tenella. Journal of Animal Research*, *53*(4): 137–144.

Dalloul, R. A. & Lillehoj, H. S. (2005). Recent advances in immunomodulation and vaccination strategies against coccidiosis. *Journal of Avian Diseases*, 49(1): 1–8.

Dalloul, R. A., Lillehoj, H. S., Lee, J., Lee, S. & Chung, K. (2006). Immunopotentiating Effect of a Fomitella fraxinea -Derived Lectin on Chicken Immunity and Resistance to Coccidiosis. *Journal of Poultry Science*, 85: 446–451.

Danforth, H.D. (1998). Use of live oocyst vaccines in the control of avian coccidiosis: experimental studies and field trials. *International Journal of Parasitology*, 28: 1099–1109.

Del Cacho, E., Gallego, M., Lee, S. H., Lillehoj, H. S., Quilez, J., Lillehoj, E. P. & Sánchez-Acedo, C. (2012). Induction of protective immunity against Eimeria tenella, Eimeria maxima, and Eimeria acervulina infections using dendritic cell-derived exosomes. *Journal of Infection and Immunity*, 80(5), 1909–1916.

Dezfoulian, O., Gharagozlou, M. J., Rahbari, S. & Samani, R. (2010). Coccidiosis due to various species of *Eimeria* in the stunted and diarrheic native turkey poults: Pathology and morphological characterization of oocysts. *Journal of Archives of Razi Institute*, 65(1):15-19.

Du, A. & Hu, S. (2004). Effects of a herbal complex against *Eimeria tenella* infection in chickens. *Journal of Veterinary Medicine*, 51: 194–197.

Gazyagci, A. N., Anteplioglu, T., Canpolat, S. & Atmaca, H. T. (2015). Coccidiosis due to *Eimeria arloingi* Infection in a Saanen Goat. *Research Journal for Veterinary Practitioners,* 3(2): 29.

Gupta, S. K. (2009). Diagnosis and control of poultry coccidiosis. *Journal of Haryana Veterinary*, 48 (11): 1–10.

Gussem, M. D. (2006). Coccidiosis in poultry: Review on diagnosis, Control, Prevention and interaction with overall gut health. *16th European Symposium on Poultry Nutrition*, 253–261.

Hafeez, M. A., Akhtar, M. & Hussain, I. (2006). Protective effect of egg-propagated *Eimeria tenella* (local isolates) gametocytes as vaccine(s) against mixed species of coccidia in chickens. *Journal of Parasitology Research*, *98*(6): 539–544.

Hafeez, M. A., Akhtar, M. & Javed, M. T. (2007). Maternal immunization by egg propagated gametocyte vaccine to control *Eimeria tenella* infection in newly hatched chicks. *Journal of Parasitology Research*, 100: 1139–1141.

Harfoush, M. A., Hegazy, A. M., Soliman, A. H. & Amer, S. (2010). Drug resistance evaluation of some commonly used anti coccidial drugs in broiler chickens . *Journal of Egypt Soc Parasitol.*, *40*(2): 337–348.

Hashemnia, M., Khodakaram-Tafti, A., Razavi, S. M. & Nazifi, S. (2014). Hematological and serum biochemical analyses in experimental caprine coccidiosis. *Journal of Parasitic Diseases*, 38(1): 116–123.

Haug, A., Thebo, P. & Mattsson, J. G. (2007). A simplified protocol for molecular identification of *Eimeria Spp* in field samples. *Journal of Veterinary Parasitology*, 146 (1-2): 35–45.

Haug, A., Gjevre, A. G., Thebo, P., Mattsson, J. G. & Kaldhusdal, M. (2008). Coccidial infections in commercial broilers: Epidemiological aspects and comparison of *Eimeria Spp* identification by morphometric and polymerase chain reaction techniques. *Journal of Avian Pathology*, *37*(2): 161–170.

Home, J., Articles, J. & Jvmah, A. (2015). Epidemiological study on poultry coccidiosis : Prevalence, Spp identification and post mortem lesions in grower chicken in Kombolcha, North-Eastern Ethiopia. *Journal of Veterinary Medicine and Animal Health*, 7(1): 18.

Idris, A. B., Bounous, D. I., Goodwin, M. A., Brown, J. & Krushinskie, E. A. (1997). Quantitative pathology of small intestinal coccidiosis caused by *Eimeria maxima* in young broilers. *Journal of Avian Pathology*, 26(4): 731–747.

Jenkins, M., Klopp, S., Ritter, D., Miska, K. & Fetterer, R., 2010. Comparison of Eimeria species distribution and salinomycin resistance in commercial broiler operations utilizing different coccidiosis control strategies. *Journal of Avian Diseases*, 54: 1002–1006.

Jiang, L., Zhao, Q., Zhu, S., Han, H., Dong, H. & Huang, B. (2012). Establishment of *Eimeria tenella* (local isolate) in chicken embryos. *Journal of Parasitology*, 19(3): 285–289.

Johnson, J. & Reid, W. M. (1970). Anticoccidial drugs: Lesion scoring techniques in battery and floor-pen experiments with chickens. *Journal of Experimental Parasitology*, *28*(1): 30–36.

Kadhim, L.I. (2014). Histopathological changes of broilers Immunized with sonicated oocysts against *Eimerria tenella*. *Journal of Advanced Biological Research*, 4(1): 31–35.

Kawazoe, U., Bordin, E. L., Lima, C. A. D. & Dias, L. A. V. (2005). Characterisation and histopathological observations of a selected Brazilian precocious line of *Eimeria acervulina. Journal of Veterinary Parasitology*, 131: 5–14.

Khan, M. N., Rehman, T., Iqbal, Z., Sajid, M. S., Ahmad, M., Riaz, M. & Area, A. S. (2011). Prevalence and Associated Risk Factors of *Eimeria* in Sheep of Punjab, Pakistan. *World Academy of Science*, 55(7): 443–447.

Kinung, S. M., Tilahun, G., Hafez, H. M., Woldemeskel, M. & Kyule, M. (2004). Assessment of Economic Impact Caused by Poultry Coccidiosis in Small and Large

Scale Poultry Farms in Debre Zeit, Ethiopia. *Journal of Poultry Science*, 3(11): 715–718.

Lee, H. A., Hong, S., Chung, Y. & Kim, O. (2011). Sensitive and specific identification by polymerase chain reaction of *Eimeria tenella* and *Eimeria maxima*, important protozoan pathogens in laboratory avian facilities. *Journal of Laboratory Animal Research*, 27(3): 255–258.

Lee, K. W., Lillehoj, H. S., Jang, S. I., Pagès, M., Bautista, D. A, Pope, C. R., Ritter, G. D., Lillehoj, E. P., Neumann, A. P. & Siragusa, G. R. (2012). Effects of in ovo vaccination and anticoccidials on the distribution of Eimeria spp. in poultry litter and serum antibody titers against coccidia in broiler chickens raised on the used litters. *Journal of Research in Veterinary Science*, *93*(1): 177–182.

Lee, K. W., Lillehoj, H. S., Jang, S. I., Lee, S. H., Bautista, D. A., Ritter, G. D., Lillehoj, E. P. & Siragusa,G. R. (2013). Comparison of live Eimeria vaccination with in-feed salinomycin on growth and immune status in broiler chickens. *Journal of Research in Veterinary Science*, *95*(1): 110–4.

Lehman, R.., Moran, E. T. & Hess, J. B. (2009). Response of coccidiostat-versus vaccination protected broilers to gelatin inclusion in high and low crude protein diets. *Journal of Poultry Science*, 88: 984–993.

Lilic, S., Tamara, I. & Sanda, D. (2009). Coccidiosis in poultry industry. *Journal of Tehnologija Mesa*, 50 (1): 90-98.

Lillehoj, H. S. & Lillehoj, E. P. (2000). Avian coccidiosis. A review of acquired intestinal immunity and vaccination. *Journal of Avian Disease*, 44(2): 408–425.

Long, P. L., Millard, B. J., Joyner, L. P. & Norton, C. C. (1976). A guide to laboratory techniques used in the study and diagnosis of avian coccidiosis. *Journal of Folia Veterinary Latin*, 6(3): 201–217.

Manyawe, S. M., .Abdel Rahman, M. A. M., Abd El, A.M.I., Kamal, Azza. M. l. & Snousi, S. A. (2010). Prevalence of Some Protozoa and Its Effects on Biochemical

Changes in Goats in Cairo, Marsa Matrouh, and El-Wadi El-Gadid Provinces. *Egypt. J. Comp. Path. & Clinic. Path*,23(1): 102–15.

Markiewicz, W., Barski, D., Tomaszewska, E. & Burmańczuk, A. (2012). Toxicity of salinomycin and narasin in turkeys. *Journal of Elemntology*, 3(1): 903–914.

Masood, S., Abbas, R. Z., Iqbal, Z., Mansoor, M. K., Sindhu, Z., Zia, M. A. & Khan, J. A. (2013). Role of Natural Antioxidants for the Control of Coccidiosis in Poultry. *Pakistan Veterinary Journal*, 33(2): 401–404.

Mathivanan, R. & Edwin, S. C. (2012). Hematological and serum biochemical parameters of broilers fed with Andrographis paniculata as an alternative to antibiotic growth promoter. *Journal of medicinal plant Research*, 6: 5647-5650.

Mcdougald, L. R. (1998). Intestinal Protozoa Important to Poultry Coccidia and Related Organisms Monitoring of Litter Oocysts. *Journal of Poultry Science*, *77*: 1156–1158.

Mcdougald, L. R., Maria, J., Da, L., Solis, J. & Braga, M. (1987). A Survey of Sensitivity to Anticoccidial Drugs in 60 Isolates of Coccidia from Broiler Chickens in Brazil and Argentina. *Journal of Avian Diseases*, *31*(2): 287–292.

Morris, G. M. & Gasser, R. B. (2006). Biotechnological advances in the diagnosis of avian coccidiosis and the analysis of genetic variation in *Eimeria*. *Journal of Biotechnology Advances*, 24(3): 590–603.

Morris, M. G., Woods, W. G., Richards, D. G., Robin, B. & Gasser. (2007). Investigating a persistent coccidiosis problem on a commercial broiler breeder farm utilizing PCR coupled capillary electrophoresis. *Journal of Parasitology Research*, 101(3): 583–589.

Mwale, M. & Masika, P. (2011). Point prevalence study of gastro-intestinal parasites in village chickens of Centane district, South Africa. *African Journal of Agricultural Research*, *6*(9): 2033–2038.

Naidoo, V., McGaw, L. J., Bisschop, S. P. R., Duncan, N. & Eloff, J. N. (2008). The value of plant extracts with antioxidant activity in attenuating coccidiosis in broiler chickens. *Journal of Veterinary Parasitology*, 153(4): 214–219.

Patra, G., Ali, M. A., Chanu, K. V., Jonathan, L., Joy, L. K., Prava, M., Ravindran, R., Das, G. & Devi, L. I. (2010). PCR Based Diagnosis of *Eimeria tenella* Infection in Broiler Chicken. *International Journal of Poultry Science*, 9 (8): 813-818.

Peek, H. W. & Landman, W. J. M. (2003). Resistance to anticoccidial drugs of Dutch avian *Eimeria Spp* field isolates originating from 1996, 1999 and 2001. *Journal of Avian Pathology*, *32*(4): 391–401.

Quiroz-castañeda, R. E. & Dantán-González, E. (2015). A review: Control of Avian Coccidiosis : Future and Present Natural Alternatives. *International Journal of Biomedical Research*. 11.

Razmi, G. R. & Kalideri, G. A. (2000). Prevalence of subclinical coccidiosis in broiler-chicken farms in the municipality of Mashhad, Khorasan, Iran. *Journal of Preventive Veterinary Medicine*, 44: 247–253.

Rizvi, F., Anjum, A. D., Khan, A., Mohsan, M. & Shazad, M. (2008). Pathological and serum biochemical effects of salinomycin on layer chicks. *Journal of Pakistan Veterinary*, *28*(2): 71–75.

Ruff, M. D. (1999). Important parasites in poultry production systems. *Journal of Veterinary Parasitology*, 84(3-4): 337–347.

Ryley, J. F., Meade, R., Hazelhurst, J. & Robinson, T. E. (1976). Methods in coccidiosis research: separation of oocysts from faeces. *Journal of Parasitology. 73*(3): 311–26.

Scanes, C. G. (2007). The global importance of poultry. *Journal of Poultry Science*, 86(5): 1057–1058.

Schnitzler, B. E., Thebo, P. L., Mattsson, J. G., Tomley, F. M. & Shirley, M. W. (1998). Development of a diagnostic PCR assay for the detection and discrimination of four pathogenic *Eimeria Spp* of the chicken. *Journal of Avian Pathology*, 27: 490–497.

Sharma, S., Iqbal, A., Azmi, A. & Shah, H. A. (2013). Study of poultry coccidiosis in organized and backyard farms of Jammu region. *Journal of Veterinary World*, 6(8): 467–469.

Sharman, P. A., Smith, N. C., Wallach, M. G. & Katrib, M. (2010). chasing the golden egg: vaccination against poultry coccidiosis. *Journal of Parasite Immunology*, *32*(8): 590–598.

Shirley, M. W., Bushell, A. C., Bushell, J. E., McDonald, V. & Roberts, B. (1995). A live attenuated vaccine for the control of avian coccidiosis : trials in broiler breeders and replacement layer flocks in the United Kingdom. *International Journal of Veterinary Research*, 137(18): 453–457.

Sivaraj, A., Vinothkumar, P., Sathiyaraj, K., Sundaresan, S., Devi, K. & Senthilkumar, B. (2011). Hepatoprotective potential of Andrographis paniculata aqueous leaf extract on ethanol induced liver toxicity in albino rats. Journal of Applied Pharmaceutical Science. 1: 204-208.

Stanley, V.G., Gray, C., Daley, M., Krueger, W.F., Sefton, A.E., 2004. An alternative to antibiotic-based drugs in feed for enhancing performance of broilers grown on Eimeria spp.-infected litter. Poultry Science, 83: 39–44.

Sultana, R., Maqbool, A., Ahmad, M., Anjum, A. A., Ch, S. I., & Ahmad, M. S. (2014). Control of Coccidiosis in Calves by Vaccination. *Journal of Bacteriology and Parasitology*, 5(4): 5.

Sun, X. M., Pang, M., Jia, T., Yan, W. C., He, G., Hao, L. L., Bentue, M. & Sue, X. (2009). Prevalence of Eimeria species in broilers with subclinical signs from fifty farms. *Journal of Avian Diseases,* 53: 301–305.

Tewari, A. K. & Maharana, B. R. (2011). Control of poultry coccidiosis: Changing trends. *Journal of Parasitic Diseases*, 35(1): 10–17.

Usman, J. G., Gadzama, U. N., Kwaghe, A. V. & Madziga, A. H. A. (2011). Anticoccidial Resistance in Poultry. A Review. *Journal of New Yark Science,* 4(8): 102– 109.

Vahdatpour, T., Nikpiran, D. H., Babazadeh, S., Vahdatpour. & Jafargholipour, M. A. (2011). Effects of Protexin®, Fermacto® and combination of them on blood enzymes and performance of Japanese quails (Coturnix Japonica). Annals of Biological Research, 2: 283-291.

Waldenstedt, L., Elwinger, K., Thebo, P. & Uggla, A. (1998). Effect of Betaine Supplement on Broiler Performance during an Experimental Coccidial Infection. *Journal of Poultry Science,* 78: 182–189.

Weber, F. H. & Evans, N. A. (2003). Immunization of broiler chicks by in ovo injection of *Eimeria tenella* sporozoites, sporocysts or oocysts. *Journal of Poultry Science*, 82(11): 1701–1707.

Weber, F. H., Genteman, K. C., Lemay, M. A., Lewis, D. O. & Evans, N. A. (2004). Immunization of Broiler Chicks by In Ovo Injection of Infective Stages of *Eimeria*. *Journal of Poultry Science,* 83: 392–399.

Williams, R. B. & Gobbi, L. (2002). vaccine and an anticoccidial drug programme in Comparison of an attenuated anticoccidial vaccine and an anticoccidial drug programme in commercial broiler chickens in Italy. *Journal of Avian Pathology*, 31*:* 253–265.

Williams, R. B. (1999). A compartmentalized model for the estimation of the cost of coccidiosis to the world's chicken production industry. *International Journal for Parasitology*, 29(8): 1209–1229.

Williams, R. B. (2002). Anticoccidial vaccines for broiler chickens: Pathways to success.*Journal of Avian Pathology*, 31: 317– 353

Windhorst, H. W. (2006). Changes in poultry production and trade worldwide. *Journal of Poultry Science, 62*(04): 585–602.

Yang, R., Brice, B., Elloit, A., Lee, E. & Ryan, U. (2015). *Eimeria collieie n. sp.* (Apicomplexa:Eimeriidae) from the western long-necked turtle (Chelodina colliei). *Journal of Experimental Parasitology*, 154: 75–81.

Yegani, M. (2009). The future of poultry science: student perspective. *Journal of Poultry Science*, *88*(6): 1339–1342.

Zhang, L., Ma, L., Liu, R., Zhang, Y., Zhang, S., Hu, C., Song, M., Cai, J. & Wang, M. (2012). Veterinary Parasitology Eimeria tenella heat shock protein 70 enhances

protection of recombinant microneme protein MIC2 subunit antigen vaccination against E . tenella challenge. *Journal of Veterinary Parasitology*, 188(3-4), 239–246.

Zhang, J. J., Wang, L. X., Ruan, W. K. & An, J. (2013). Investigation into the prevalence of coccidiosis and maduramycin drug resistance in chickens in China. *Journal of Veterinary Parasitology*, 191(1-2): 29–34.