EFFICACYAND IMMUNOGENICITY OF *EIMERIA TENELLA* APICAL MEMBRANE ANTIGEN-1 (EtAMA1) DNA VACCINE AGAINST *EIMERIA TENELLA* INFECTION IN BROILER CHICKENS

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APRIL, 2021

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DVM (ABU) 2008
(P17VTVM8004)

A THESIS SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, AHMADU BELLO UNIVERSITY ZARIA, NIGERIA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF MASTER OF SCIENCE IN AVIAN MEDICINE

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APRIL, 2021

DECLARATION

I declare that the work in this Dissertation entitled "**Efficacy and Immunogenicity of *Eimeria tenella* Apical Membrane Antigen-1 (EtAMA1) DNA VaccineAgainst *Eimeria tenella* Infection in Broiler Chickens**" has been carried out by me in the Department of Veterinary Medicine, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria under the supervisions of Prof. P.A. Abdu and Dr. I.D. Jatau. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this Dissertation was previously presented for another degree, diploma or certificate at any institution.

Paul Terkende HAMBESHA ………………… ……………………

Signature Date

CERTIFICATION

This Dissertation titled "**EFFICACY AND IMMUNOGENICITY OF *EIMERIA TENELLA* APICAL MEMBRANE ANTIGEN-1 (EtAMA1) DNA VACCINE AGAINST *EIMERIA TENELLA* INFECTION IN BROILER CHICKENS**" by Paul Terkende HAMBESHA meets the regulations governing the award of the degree of Master of Science of the Ahmadu Bello University Zaria, and is approved for its contribution to knowledge and literary presentation.

Prof. P. A. Abdu	…………………….	………………...
Chairman, Supervisory Committee	Signature	Date

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DEDICATION

This work is dedicated to the Almighty God our heavenly Father from whom all life and goodness comes.

ACKNOWLEDGEMENTS

Unto you, the Almighty God, king of eternal glory, source of my being and keeper of my soul, I give all my praises and adoration for giving me the grace to accomplish this study. With love I express my profound gratitude to those that have contributed to make this work a success. So much thanks to the Chairman of my supervisory committee, Prof. P. A. Abdu for the immense support of my MSc study and research demonstrated via his patience, motivation, enthusiasm, and vast knowledge. His guidance helped me on how to carry out research and to present the research work as clearly as possible. It was a great privilege and honour to work and study under him. I could not have imagined having a better advisor and mentor for this study.

To my second supervisor, Dr. I. D. Jatau who also supported and taught me the methodology to carry out the research and through whom I got invaluable materials like the DNA vaccine and Houghton strain of *Eimeria tenella* from the Department of Pathology and Pathogen Biology of the Royal Veterinary College, UK. I am extremely grateful. His friendship and empathy have deeply inspired me and I deeply appreciate.

I am highly obliged and thank Prof. C.A. Kudi, Prof. L. Saidu, Prof. J. Kabir, Prof. A.M. Wakawa, Dr. P. Mamman, Dr. G. Kia and Dr. S. J. Enam for their contributions in this study. I sincerely thank Dr. B. Yakubu (Head of Biotechnology Laboratory, NVRI, Vom), who did not only grant me a Bench Space at NVRI, Vom, but together with Dr. Benshak J. Audu and Dr. Luka Pam supported me in the laboratory and guided me in purchasing items like the Primers and plasmid DNA purification kits. In a unique way, I cherished so much the Statistician, Dr. Ochuko Orakpoghenor, who analysed my experimental data. I equally appreciate Prof. Damer P. Blake of RVC, University of London who donated the DNA vaccine and the Houghton strain of *Eimeria tenella* used in this research.

Also, I appreciate the following people:Mr. Edima O. Obaja, Mallam Iliya Sale, Mr. Leo David, Mal. Yusufu Magaji, Late Alhaji Sale, Pst Zaphaniah N. Jangber and Mal. Aliyu Yakubu who supported me in diverse ways during the course of the research.

Furthermore, I am thankful to all my postgraduate lecturers and friends who gave me support and encouragement in the course of this study. I appreciate all my postgraduate colleagues, classmates: Drs P. Mosimabale, B. Okaiyeto, R. Nasiru, C. Udechuckwu, I. Abdulmajeed, H. Bitrus, M. Usman, M. Damina, J. Amabo and K. Jolayemi for every support I received, without which completion of this project could not have been accomplished.

My unreserved honour goes to my parents: Mr. and Mrs S.A Hambesha for laying my educational foundation and still nurturing it even at this stage; brothers, sisters and all my relations who supported me directly or indirectly in this research. To my children:

Vershima, Nguvan, Mdoommater and Shaana; I thank them for allowing me time away from them to carry out research and write. They deserve a trip to Disney!

To my caring, loving, and supportive wife, Rosemary (ADOOTERSHIMA): my deepest gratitude. Your encouragement and ideas when the times got rough are much appreciated and duly noted. It was a great comfort and relief to know that you were willing to provide management of our household activities while I left for study. I am ever grateful.

I have no valuable words to express my thanks, but my heart is still full of the favours received from everyone.

ABSTRACT

Apical membrane antigen-1 (AMA1), being a micronemal transmembrane protein of apicomplexan parasites, is secreted by the microneme organelles onto the parasite surface during host cells invasions. In this study, the performances, immune response, haematological, gross, and histopathological changes were assessed to evaluate the level of protective immunity of the *Eimeria tenella* apical membrane antigen-1 (EtAMA1) DNA vaccine in broiler chickens challenged with two strains of *E. tenella* oocysts. Eighty (80) day-old broiler chicks divided into four groups (G1, G2, G3, and G4) were used for the study. Chicks in G1 were unvaccinated unchallenged and G2 unvaccinated challenged. Chicks in G3 and G4 were intramuscularly vaccinated with a DNA vaccine, a construct of EtAMA1 Houghton strain, at 14, and21daysofage. Seven days after the booster vaccination, G2 and G3 were challenged with a local isolate of *E. tenella* while G4 was challenged with Houghton strain of *E. tenella*. The chickens were observed for clinical signs, body weight gain, survival rate, oocyst count, lesion scores, and anticoccidial index. Blood samples were collected on day 14, 21, 28, and 35 of age for haematology and sera harvested for determination of IgY and interferongamma (IFN-γ) concentration. Also, at 7 days post challenge, caeca collected from sacrificed birds were observed grossly for lesions, weighed and fixed in 10 % neutral buffered formalin for histopathology. Clinical signs associated with coccidiosis which include depression, dullness, somnolence, reduced feed in-take, bloody faeces, and soiled vent were mild in vaccinated challenged chickens, but very severe in unvaccinated challenged chickens.There was significant ($p < 0.05$)reduction in the oocyst output and increased weight gain in vaccinated challenged birds (G3 and G4) compared to unvaccinated challenged birds (G2). Haematology revealed significantly (p <

0.05) decreased PCV, Hb, total protein, MCV, MCH, and MCHC in unvaccinated challenged chickens when compared to vaccinated chickens. The heterophil/lymphocyte ratio was significantly higher in unvaccinated challenged birds. Significantly high ($p < 0.05$) levels of serum IgY and IFN-γ concentrations were observed in vaccinated chickens. Histopathological examination showed desquamation and necrosis of mucosal glands in unvaccinated challenged birds. The results showed that DNA vaccine encoding EtAMA1 improved performance and elicited an increase in serum levels ofIgY and IFN-γ thus, could provide some level of immunity against caecal coccidiosis of not just that of the homologous strain but also of a heterologous strain from another geographical area.

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LIST OF ABBREVIATIONS AND SYMBOLS

%	Per cent
$	US dollar (s)
µg	Microgram
µl	Microlitre
µm	Microns
ABU	Ahmadu Bello University
ACI	Anti-coccidial index
ACP	Anticoccidial products
Ad-lib	*ad libitum*
AFLP	Amplified fragment length polymorphism
AMA1	Apical membrane antigen-1
ANOVA	Analysis of variance
ASTs	Anticoccidial sensitivity tests
Bp	Base pair
CO_2	CarbonIV oxide
DNA	Deoxyribonucleic acid
DOC	Day-old-chick
dpi	Days post inoculation
E	East of the Greenwich Meridian
E.	*Eimeria*
ELISA	Enzyme linked-immunosorbent assay
EtAMA1	*Eimeria tenella* apical membrane antigene-1
g	Grams
GDP	Gross domestic product

H & E	Haematoxylin and Eosin
h	Hour (s)
Hb	Haemoglobin
IFN-γ	Interferon-gamma
IgY	Immunoglobulin Y
m^2	Metre square
MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobin concentration
MCV	Mean corpuscular volume
ml	Millilitre (s)
mm	Millimetre (s)
N	North of the Equator
^oC	Degree Celsius
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCV	Packed cell volume
RBC	Red blood cells
SCAR	Sequence characterized amplified region
SEM	Standard error of the mean
SPSS	Statistical Package for Social Science
TLR	Toll-like receptors
WBC	White blood cell

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of the Study

Coccidiosis is one of the major infectious poultry diseasesworldwide. The disease is caused by an obligate intracellular protozoan known as *Eimeria* of the phylum Apicomplexa(Brown-Jordan *et al.,* 2018).Of the seven *Eimeria* species widely recognized as the causative agent of coccidiosis in chickens, *E. tenella, E. necatrix, E. maxima*and *E. brunetti* are highly pathogenic; *E. acervulina* and *E. mitis* are less pathogenic; while *E. praecox* is regarded as the least pathogenic (McDougald, 2003; Conway and McKenzie, 2007; Taylor *et al.,* 2007). All of these species have been detected in Nigeria (Bishu, 1982; Jatau *et al.,* 2012; 2016; Agishi *et al.,* 2016).

Prevalence of coccidia infection vary between 10% and 90% in industrial chicken farms worldwide (Guven *et al*., 2013).Chickens are economically significant poultry species due to their high protein content, short generation interval, and global acceptability (Blevin *et al.,* 2018; Qi *et al.,* 2018). However, the subclinical and clinical effects of coccidiosis result in malabsorption, reduced feed conversion rate and weight gain, and an increase in mortality and susceptibility to secondary disease in infected chickens (Lillehoj *et al.,* 2004; Seifert, 2006; Lawal *et al.,* 2016; Prakashbabu *et al.*, 2017).

Good animal husbandry such as maintaining litter quality, controlling house temperature, ventilation, humidity and thorough cleaning between flocks are means to prevent and control coccidiosis because these measures reduce oocysts sporulation and consequently decrease the parasite’s population (Blake and Tomley, 2014). Notwithstanding, prophylactic use of anticoccidial drugs has been the primary means of controlling chicken coccidiosis in broiler industry and has played a major role in the growth of the industry (Kitandu and Juranová, 2006).

The challenge of drug resistance of *Eimeria*species and cost of vaccine production have prompted major research efforts to seek alternative means of control through increased knowledge of understanding the immunomodulation, natural-product feed additives, and advances in live and recombinant vaccines (Kitandu and Juranová, 2006). The quest for these more cost-effective vaccines against coccidiosis for the mass broiler market has led to the discovery of various subunit vaccine candidates one of which include the apical membrane antigen-1 (AMA1). This is a micronemal protein of apicomplexan parasites, a transmembrane protein that is secreted from the microneme organelles that appears to be essential during the entry into the host's cells by the invasive stages of the parasites (Besteiro *et al.*, 2009; Santos *et al.*, 2011; Lamarque *et al.*, 2011; Curtidor *et al.*, 2011; Jiang *et al.*, 2012). It was first identified as a conserved antigenic protein in the malaria parasite, *Plasmodium knowlesi* (Thomas*et al.*, 1984).Blake *et al.* (2011) and Jiang *et al.* (2012) reported that *Eimeria tenella*apical membrane antigen-1(EtAMA1) have conferred significant immune protection against experimentally infected broiler chickens with *E. tenella*.

Coccidiosis in chickens is endemic in Zaria and outbreaks due to the highly pathogenic*E. tenella* and *E. necatrix* are common (Bishu, 1982; Abdu *et al.*, 2008; Musa *et al.*, 2010; Jatau *et al.*, 2012).

1.2 Statement of the Research Problems

Epidemiological studies in Nigeria have shown the economic importance of coccidiosis as a major parasitic disease of poultry (Majaro, 1980, 1983, 2001; Adene and Oluleye, 2004;Etuk *et al.,* 2004; Abdu, 2007;Musa *et al.,* 2010; Usman *et al.,* 2011; Agishi *et el*., 2016) and*E. tenella* is the commonest and major cause of haemorrhagic coccidiosis in poultry establishments worldwide, causing high morbidity and variable mortalities (Taylor *et al.*, 2007; Jatau *et al.*, 2012).

The poultry industry contributes up to 15 % to Nigeria's gross domestic product (GDP) and accountsfor 36% of total protein intake (Akintunde *et al.*, 2015), but diseases such as coccidiosis are a major limiting factor (Perry *et al.*, 2002; Haug *et al.*, 2008a; Kumar *et al.*, 2014). Consequently, the livestock industry suffers a global loss exceeding $3 billion dollars, making precautions to control the disease fundamental (Cheng *et al.*, 2018). Additional risk has been noted in much of the developing world where *Eimeria* can undermine the rapid expansion of poultry production and exert a profound impact on local poverty (Perry *et al.*, 2002).

Chemoprophylactic and chemotherapeutic treatment so far remains the most practiced means of controlling coccidiosis, but the increase in population of resistant parasite and public pressure to limit the use of chemicals in food animals poses a challenge (Williams, 2002;Abdu *et al.*, 2008; Blake*et al.*, 2011a). Resistance to anticoccidial drugs has been recognised for decades and regarded as ubiquitous, with wide acceptance that drugs remain effective in the field only because they suppress parasite growth sufficiently to allow the birds to develop natural immunity (Chapman, 1999).

There are concerns about drug residues inpoultry products (McEvoy, 2001; Young and Craig,2001); and there is a strong desire by health authorities and consumers toban drugs from animal feeds (Young and Craig, 2001). Recent regulatorycontrols by the European Union have already forcedthe withdrawal of several brands of anticoccidialdrugs commonly used in broiler shuttle programmes(Farrant, 2001). There is, therefore, a pressing need to move away from chemotherapeutic control of coccidiosistowards vaccination. Price *et al.* (2016), Ritzi *et al.* (2016), and Jenkins *et al.* (2017) demonstrated the effectiveness and potential of live anticoccidial vaccines in alleviating *Eimeria* oocyst output in broiler chickens. However, live vaccines are required to be administered within the chicken itself resulting in a low reproductive index of attenuated vaccine pathogens. This, in turn, increases production costs and limits the efficacy of the vaccine (Tan *et al.*, 2017).

1.3 Justification of the Study

The search for a sustainable cost effective vaccine against coccidiosis that will be attractive to all poultry sectors has so far resulted in the identification of novel recombinant vaccine candidates, among which AMA1 has great potential of becoming a more realistic candidate (Jatau, 2014; Blake *et al.*, 2015). Under experimental conditions, studies with clonal reference strains of *Eimeria*have found AMA1 to induce good levels of immune protection in challenged broiler chickens (Blake *et al.*, 2011a; Jiang *et al.*, 2012), making the development of recombinant AMA1 or vectored subunit anticoccidial vaccine technically more realistic and supporting the validity of AMA1 antigen to confer universal protection against field strains of *Eimeria* species when used in future vaccine development (Jatau, 2014; Blake *et al.*, 2015). Therefore, the need for DNA and recombinant vaccines has become imperative (Pastor-Fernández *et al.*, 2018).

However, for any novel subunit anticoccidial vaccine to proceed beyond experimental phase, evidence of likely field efficacy against novel strains is essential. To this end, Shuaibu (2018) did a comparative work on the efficacy of the DNA vaccine and live oocysts vaccines against experimental infection with a local isolate from the National Veterinary Research Institute (NVRI), Vom, and Houghton strain of *E. tenella* in broilers and observed that, the monovalent AMA1 DNA vaccine was able to induce some level of immune protection against the wild strain of *E. tenella*. The researcher recommended immunological studies to be conducted to determine the nature and type of immune response.

1.4 Aim of the Study

The aim of the study was to evaluate the efficacy, and immunogenicity of *E. tenella*apical membrane antigen-1 (EtAMA1) DNA vaccine against *E. tenella* infection in broiler chickens.

1.5 Objectives of the Study

The objectives of the study were to:

i. Evaluate the effect of immunization with EtAMA1 DNA vaccine on clinical signs, body weight gain, feed conversion ratio, survival rate and oocyst count in broiler chickens experimentally infected with *E. tenella* local isolate or Houghton strain.

ii. Evaluate the effects of immunization with EtAMA1 DNA vaccine on some haematological parameters in broiler chickens experimentally infected with *E. tenella*local isolate or Houghton strain.

iii. Determine the immune response induced by EtAMA1 DNA vaccine in broiler chickens experimentally infected with *E. tenella*local isolate or Houghton strain.

iv. Assess the effects of immunization with EtAMA1 DNA vaccine on gross, and histopathological changes in the caeca of broiler chickens experimentally infected with *E. tenella* local isolate or Houghton strain.

1.6 Research Questions

i. Does vaccination with EtAMA1 DNA affect the clinical signs, body weight gain, feed conversion rate, survival rate and oocyst count in broiler chickens experimentally infected with *E. tenella* local isolate or Houghton strain?

ii. Does EtAMA1 DNA vaccine have effect on some haematological parameters of broiler chickens experimentally infected with *E. tenella*local isolate or Houghton strain?

iii. Does EtAMA1 DNA vaccine induce protective immune response in broiler chickens experimentally infected with *E. tenella*local isolate or Houghton strain?

iv. What are the effects of immunization with EtAMA1 DNA vaccine on gross, and histopathological changes in the caeca of broiler chickens experimentally infected with *E. tenella*local isolate or Houghton strain?

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Poultry Coccidiosis

2.1.1 Background information

Poultry coccidiosis is an intestinal parasitic disease caused by an obligate intracellular protozoan of the genus *Eimeria*. (McDonald and Shirley, 2009; Blake and Tomley, 2014; Brown-Jordan *et al.*, 2018). 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; Prakashbabu *et al.*, 2017). The induced tissue damage and change in intestinal function may allow colonization by various harmful bacteria, such as *Clostridium perfringens*, leading to necrotic enteritis (Helmboldt and Bryant, 1971).Poultry coccidiosis may affect any type of poultry in any type of facility (McDougald, 2003). The synonyms of poultry coccidiosis are: Coxy,*Eimeriasis* and “Brooder house coccidiosis” or “Bloody coccidiosis” (Abebe and Gugsa, 2018; Abdu and Musa, 2019).

2.1.2 History of poultry coccidiosis

At the beginning of the 20th century, it was not clear as to the organism that caused the disease coccidiosis in poultry and other agricultural livestock. Railliet and Lucet (1891) observed oocysts, as well as the transmission stage of the parasite in caeca of a chicken and conducted the first experimental infections. They named the parasite *Coccidium tenellum* but subsequently changed it to *Eimeria tenella*(Railliet, 1913). Fantham was the first to provide a detailed description of the life cycle of an *Eimerian* parasite in an avian host. The researcher was employed to investigate mortality in Red Grouse (*L. lagopus*) on the grouse moors of Scotland,the outcome was a parasitic isolate named *Eimeria avium* (Fantham, 1910). Fantham recorded key aspects of the biology of these organisms including the liberation of sporozoites from the sporocysts, cell penetration by sporozoites, schizogony and merozoite formation (asexual multiplication), formation of macro- and microgametocytes (sexual reproduction), oocyst wall formation, and sporogony (spore formation). Fantham provided the first detailed drawing of the life cycle illustrating these phases of development (Chapman, 2003). The intestinal phases of the life cycle have been confirmed in many other studies, and an extra intestinal phase, involving sporozoite migration to the spleen and liver, has also been observed (Fernando *et al.,* 1987). Significant progress in the understanding of coccidiosis was made by Johnson (1923) whose contrary opinion was that, coccidia were not responsible for blackhead disease (subsequently shown by Tyzzer (1932) to be caused by the flagellate *Histomonas meleagridis* and various other pathological conditions that affected the fowl).The author pointed out that infection is self-limiting, that the severity of coccidiosis is proportional to the numbers of sporulated oocysts ingested, and that transmission within the egg was improbable. Also, that a species of *Eimeria*that infects chickens was unable to infect other avian hosts, thus demonstrating the phenomenon of host specificity, and he equally noticed distinct variations in the size and shape of oocysts suggesting that more than one species might occur in the chicken. Johnson (1923) was the first to investigate the acquisition of immunity and showed that resistance to a challenge dose of oocysts was not due to age but dependent on prior exposure to infection. The scientist anticipated the possibility of vaccinating chickens by infecting them with live oocysts and instigated experiments to immunize birds by incorporating repeated small doses of oocysts in the feed (Johnson, 1927). He was also the first to observe *Eimeria praecox* and *Eimeria necatrix*, which were independently discovered and described in detail by Tyzzer *et al.* (1932). Johnson's many contributions to the basic understanding of coccidiosis have been reviewed (Chapman, 2004). Tyzzer *et al.* (1932) described the lesions in the intestines associated with acute infection therefore, providing much information on morphological characteristics, pathogenicity, host specificity, immunogenicity, and pathology. His work is the platform upon which modern understanding of coccidiosis is based.

2.1.3 Epidemiology of poultry coccidiosis

2.1.3.1 Distribution

Coccidiosis is a disease common in intensively managed farms especially where management or hygiene standards are compromised (Musa *et al.,* 2010).Coccidia are distributed worldwide in poultry and wild birds and almost universally present in poultry raising operations (Larry and Otto, 2005).

2.1.3.2 Incidence

Poultry coccidiosis occurs all year round, but generally more frequently during rainy season than dry season of the year (Murry and Hanson, 2005). The incidence rate of coccidiosis is positively influenced by the warm and humidweather, which characterizes the rainy season period by providing favourable conditions for the development of the infective oocysts (Etuk *et al*., 2004; Alawa *et al*., 2010). Obasi *et al*. (2001) reported that exposure potential of chicken to coccidia parasites in warm humid tropical condition is high, since environmental conditions favour an all year round development of the coccidia organisms. Birds managed on deep litter show higher incidence of coccidiosis perhaps due to their close contact with the infective oocysts in the litter (Etuk *et al*., 2004). Similarly, high stocking density was reported to facilitate coccidiosis (Trees, 1999; David, 2000; Etuk *et al*., 2004).

Sudden outbreak of coccidiosis occurs following the ingestion of high doses of the sporulated oocysts over a short period of time by non-immune young (3-8 week old) birds (Musa *et al.*, 2010). Birds of any age are susceptible to coccidiosis but most birds get infected in the early few weeks of life (Chookyonix *et al.*, 2009). Coccidiosis has been reported about 3 days following ingestion of large numbers of sporulated oocysts (Urquhart *et al.*, 1996) and under field condition, the incubation period for intestinal coccidiosis was reported to be 5 days while that of caecal coccidiosis was 5-6 days (Chookyonix *et al.*, 2009; Musa *et al.*, 2010). Additionally, oocysts have been practically shown to survive outside the host for up to 2 years and resisted low temperatures, dry conditions and many forms of disinfectants (David, 2000).

2.1.3.3 Transmission

Transmission of poultry coccidiosis is through faeco-oral route (Chapman *et al*., 2002; Nematullahi *et al.*, 2008). It is transmitted by directand indirect contact with the droppings of infected birds. Coccidiosis could also be spread between birds by the consumption of food or drinking water contaminated by faeces containing the infective stage of the coccidia which are known as sporulated oocysts (Chapman *et al*., 2002).Oocysts are distributed within the poultry building, inside and outside the house by invertebrates and vermin whilst mechanical ventilation systems serve to scatter the oocysts outside the house (Taylor *et a*l., 2007).

Fecal contamination of vehicles and personnel can spread the infection to other farms.People are important vectors of coccidia in disseminating oocysts, which could be carried over by manure clinging to shoes or by utensils carried about from one pen or farm to the other (Charlton, 2006; Nematullahi *et al.,* 2008). Flies, beetles, cockroaches, rodents, pets and other birds have also been incriminated as mechanical vectors (Charlton, 2006; Abdu *et al*., 2008; Musa *et al*., 2010).

2.1.4 Prevalence of poultry coccidiosis

Coccidia prevalence has been reported in all flocks worldwide (Muazu *et al.*, 2008;Yu-wen, 2009; Györke *et al.,* 2013; Gharekhani *et al.*, 2014). Guven *et al.* (2013), reported the prevalence of coccidia infection to vary between 10% and 90% in industrial chicken farms worldwide. The global prevalence of *Eimeria*-infected flocks has been estimated at more than 50% in countries throughout Asia, Europe, the Middle East, and South America (Haug *et al.,* 2008b; Sun *et al.*, 2009; Nematullahi *et al.,* 2009; Lee *et al.*, 2010; Brooks-Pollock *et al.*, 2015). Muazu *et al.* (2008) reported an overall 52.9% prevalence of coccidiosis across the 36 states and the federal capital territory Abuja in Nigeria. The prevalence of coccidiosis is higher during the rainy season, primarily because it is positively influenced by the warm and humid weather, which characterizes the rainy season period by providing favourable conditions for the growth and development of the infective oocysts (Etuk *et al.*, 2004; Alawa *et al.,* 2010). The disease occurs throughout the year in northern Nigeria but with higher prevalence rates from May to September (Abdu, 2007). Outbreaks of up to 50% mortality were reported in commercial poultry in Zaria (Abdu *et al.*, 2008; Musa *et al.*, 2010).

Coccidiosis is also most prevalent among young chicks of 1-5 weeks of age as oocysts could appear in faecal samples of birds as early as 7 days of age with the clinical disease manifesting by the 4^{th} week (Majaro *et al.*, 2001; Obasi *et al.*, 2001). Obasi *et al.* (2001) reported that exposure potential of chicken to coccidia parasites in warm humid tropical condition is high, since environmental conditions favour an all year round development of the coccidian organisms.

2.1.5 Aetiology of poultry coccidiosis

Poultry coccidiosis is caused byan obligate intracellular protozoan parasite of *Eimeria* species in the kingdom Protozoa, phylum Apicomplexa, class Coccidia, order Eucoccidiorida, family Eimeridae and genus *Eimeria* (Taylor *et al*., 2007; Brown-Jordan *et al.,* 2018). Seven different species of this single celled parasite in the epithelial cell of the intestine of almost exclusively the genus *Eimeria (E. acervulina, E. brunetti, E. maxima, E. mitis*, *E. necatrix*, *E. praecox* and *E. tenella*) are recognized as infecting chickens (Alberta, 2007; Patrick and Mgbere, 2010; Lai *et al.*, 2011). Recently, three other species of*Eimeria* infecting chickens have been discovered namely, *E. lata*, *E. nagambie* and *E. zaria* (Blake *et al*., 2021).In Nigeria, six of these species have been identified: *Eimeria tenella*, *E.*

necatrix, E. brunetti, E. acervulina,E. mitis, and *E. praecox, an*(Bishu, 1982; Owai and Mgbere, 2010; Jatau *et al.,* 2012). The two most pathogenic species however are *E. tenella*and *E. necatrix*(Jatau *et al*., 2012), but mixed infections are commonly observed resulting in lower meat and egg productions (McDougald and Reid, 1997; Maikai *et al.*, 2007). Infection with a single species of coccidia are rare in natural conditions, mixed infection being the rule but nevertheless in many outbreaks, the clinical entity can be ascribed principally to one species occasionally a combination of two or three (Macmullin, 2001; Patrick and Mgbere, 2010).

2.1.6 Identification and taxonomy of *Eimeria*

The protistan phylum Apicomplexa (Levine, 1970) contains many obligate intracellular parasites and includes such diverse organisms as coccidia, gregarines, piroplasms, haemogregarines and the malarias. Members of this large, heterogeneous assemblage are united, not necessarily by their biology and/or life histories, but by the presence of a unique "apical complex," composed of polar rings, rhoptries, micronemes, often a conoid, and other subcellular organelles, but visualized only by use of an electron microscope (Levine *et al*., 1980).

Traditional methods of species identification have involved assessment of several phenotypic characteristics such as site of development, cross-immunity, characteristic lesions, and pathogenicity (Joyner and Norton, 1978); these are time consuming and expensive. Although still necessary, they are now complemented by molecular methods that involve PCR diagnostic assays based on DNA amplification. MacPherson and Gajadhar (1993) employed arbitrary primers under low stringency and Stucki *et al.* (1993) developed a specific PCR assay based on *E. tenella*5S rDNA. This has been improved by assays capable of detecting and differentiating all 7 species that infect chickens using the internal transcribed spacer 1 regions of rDNA (Schnitzler *et al.,* 1998). These contain sufficient interspecific variation to enable the selection of primers that can be used in PCR analyses to differentiate species (Schnitzler *et al.,* 1998). An alternative recently developed by Gruber and colleagues involves the conversion of anonymous random amplified polymorphic DNA markers into sequence-characterized amplified region markers (Fernandez *et al.,* 2003). A set of molecular (PCR-based) and computational tools involving morphological characteristics (COCCIMORPH) has been developed for species differentiation (Castanon *et al.,* 2007). Early work on the taxonomy of *Eimeria*and their relationship to other Apicomplexa was based on morphological and biological characteristics (Levine *et al.,* 1980). Characterization of small subunit (18S) rDNA gene sequences by Barta and colleagues has allowed the phylogenetic relationships of *Eimeria*species infecting the fowl to be inferred and compared with other Apicomplexa (Barta *et al.,* 1997). A maximum likely-hood consensus tree was constructed from many *Eimeria*species and it has been shown that the clade of eimeriid coccidia is not monophyletic, indicating that the taxonomy of this genus is far from resolved (Chapman *et al.,* 2013).

2.1.7 Biochemistry of *Eimeria*

The work of Wang and colleagues in the 1970s indicated various unique aspects of the biochemistry of avian coccidia and the mode of action of several anticoccidial drugs widely used at the time by the poultry industry (Wang, 1978). He noted the difficulty of isolating viable schizonts or gametocytes and obtaining pure preparations free of host and bacterial contamination, which hindered progress in understanding the biochemistry of intracellular life cycle stages. However, oocysts could be purified in large numbers and it was possible to investigate their structure and biochemical events that occur during sporogony. The oocyst wall was shown to have two layers, the inner layer a glycoprotein (Stotish and Wang, 1977) and the outer layer n-hexacosanol, a C-26 primary fatty alcohol (Weppelman *et al.,* 1976). Wang predicted that the inner layer comprised a network of small peptides interlinked by disulfide bonds (Wang, 1978). Belli and colleagues have shown that during development of the oocyst wall two tyrosine-rich proteins derived from the wall-forming bodies of macrogamonts are processed into smaller glycoproteins. They are then cross-linked to form dityrosine matrices that are believed to contribute to the resilience of the oocyst and its survival in the environment (Belli *et al.,* 2003). Recently, a sugar polymer β-1, 3-glucan has been found to form a porous trabecular scaffold in the inner wall of oocysts of *Eimeria*(Bushkin *et al.,* 2012). The outer wall of the oocyst was shown to contain a complex set of triglycerides rich in long fatty acyl chains that form a waxy coat of acid-fast lipids that confer environmental resistance (Bushkin *et al.,* 2013).

2.1.8 Classification of poultry coccidiosis

2.1.8.1Caecal and intestinal coccidiosis

Poultry coccidiosis outbreaks are commonly classified according to the areas of the digestivetract for which the particular species of coccidia seem to have a special affinity.

For practical consideration, the disease is often known as either caecal or intestinal coccidiosis (Abebe and Gugsa, 2018).

2.1.8.1.1 Caecal coccidiosis

Caecal coccidiosis is caused by only one of the species (*E. tenella*) of coccidia (Chapman, 2004). This type of coccidiosis is frequently referred to as "brooder-house coccidiosis" or "bloody coccidiosis"(Abebe and Gugsa, 2018).Caecal coccidiosis is characterized by a bloody diarrhoea, which is due to the damage done by the coccidial parasite to the mucous membrane of the caeca and rectum (Abebe and Gugsa, 2018). In the average commercial poultry flock, caecal coccidiosis is most likely to occur during the brooding period (Musa *et al.,* 2010). It is observed most often in chicks between 4 and 10 weeks of age (Musa *et al.,* 2010). Susceptible chickens younger and older than this, however, may become infected (Majaro *et al.,* 2001; Obasi *et al.,* 2001). In most outbreaks the condition appears quite suddenly, and blood in the droppings may be noted before many of the chicks show symptoms (Musa *et al.,* 2010). Following the blood in the droppings, usually within a few hours, the infected chicks lose their appetite and become droopy(Abebe and Gugsa, 2018). Death soon follows in most chickens that are severely infected. The mortality following an outbreak of caecal coccidiosis may vary from a few birds to as much as 25 per cent or more(Abebe and Gugsa, 2018). This variation depends upon several factors but especially upon the management condition under which the chicks are being kept. The sudden onset of the disease with bloody diarrhoea from the chicks is quite indicative of caecal coccidiosis. Post-mortem examination of sick or dead chicks that reveal the caeca distended with blood is practically certain to be caecal coccidiosis(Abebe and Gugsa, 2018). Some chicks that have survived an infection for several days may have the caeca filled with a cheese like "plug." These "plugs" are organized blood clots and may have a pinkish colour on the inside when cut open (Jatau *et al*., 2012; Abdu and Musa, 2019). The presence of reddish or orange-colored slimy droppings should not be confused with pure blood in the droppings because this type of dropping may be passed by chickens that are not infected with coccidia (Abdu and Musa, 2019). Microscopic examination of caecal contents and scrappings is the most dependable means of positively diagnosing caecal coccidiosis (McDougald andHu, 2001; Majaro *et al.*, 2001; Obasi *et al.*, 2001; Abebe and Gugsa, 2018).

2.1.8.1.2Intestinal coccidiosis

Intestinal coccidial infections may be caused by a single species or any combination of the six species (*Eimeria acervulina,E. brunetti, E. maxima, E. mitis, E. necatrix,* and*E. praecox*) of coccidia known to infect the intestinal tract of chickens (Abebe and Gugsa, 2018). The lesions that characterize this type of coccidiosis depend on the species of coccidia causing the disease. It should be understood, however, that the lesions and symptoms of intestinal coccidiosis are not peculiar to this condition only and may be confused with lesions caused by other diseases. One of the species of coccidia causes a type of condition in the intestinal mucosa that has been referred to as "chronic coccidiosis" (Abebe and Gugsa, 2018). The thickened spongy appearance of the mucosa with whitish patches of colonies of oocysts (*E. acervulina*) presents gross lesions mistakenly termed "chronic." "Fade-out" or "going light" is a common symptom of intestinal coccidiosis, although this reaction does not necessarily infer a "chronic" condition. It has been demonstrated experimentally that chickens severely infected with some of the intestinal coccidia may lose body weight. This loss, however, is usually regained within a short period of time. Experimental evidence indicates that the term "chronic coccidiosis" is misleading insofar as the coccidial infection is concerned. One of the most consistent conditions observed with intestinal coccidiosis is the production of a quantity of slimy mucoid or catarrhal exudate in the intestinal tract, which is frequently passed in the droppings and may form a cast around the small intestinal faeces. Haemorrhage of varying degrees may be observed from the intestinal mucosa as a result of infection with at least two of the small intestinal species *(E.maxima* and *E.necatrix*). Small pinpoint or petechial haemorrhages seen through the serosal wall of the intestine are suggestive of this type of coccidiosis. Severely infected regions of the small intestine are usually dilated, and the lumen is filled with foul-smelling, bloody mucoid content. Intestinal coccidial infections are most commonly encountered in young pullets within a few weeks after they are confined to the laying hous*e.* Intestinal coccidiosis, however, may prove to be a serious factor in susceptible birds of any age. There are no characteristic or suggestive signs such as the bloody droppings of caecal coccidiosis to warn the poultry farmer of intestinal coccidiosis (McDougald, 2003; Abebe and Gugsa, 2018). Recent studies indicate that the most suggestive signs of this type of coccidiosis are the effect on food consumption and egg production. Pullets normally come into egg production with a regular increase in rate of lay until they have reached a peak that is held more or less uniformly. When all management factors appear normal and the pullets either do not show the regular increase or if they drop rather sharply from the peak production, intestinal coccidiosis may be the responsible factor. Unfortunately, such signs are merely indicative and may be caused by a number of different disease-producing agents. Although signs and lesions may be suggestive, a microscopic examination of scrapings from the intestinal mucous membrane of two or three sick birds by a qualified person, for coccidial forms, should be the criterion for making a definite diagnosis of small intestinal coccidiosis. A positive diagnosis should not be made unless the number of coccidia present, the history of the flock, and the lesions indicate a severe coccidial infection. Birds selected for postmortem examination should be those that have just started to show symptoms. Birds that have been sick for several days or weeks and are badly emaciated or light in body weight are likely to have passed most of the parasites in the droppings and a definite diagnosis may be questionabl*e* (Allen and Fetterer, 2002; Abebe and Gugsa, 2018).

2.1.9 Life cycle of *Eimeria*

Eimeria life cycle is complex and comprises of three stages, one occurs on litter under the conditions of humidity, adequate temperature, and oxygen supply (sporogony), and two stages occurs in the cells of intestinal epithelium [merogony or schizogony (asexual reproduction) and gametogony (sexual reproduction)] (Schito and Barta, 1997; Lillehoj *et al.,* 2011).

During sporogony, which is considered a non-infective stage, the oocyst is excreted in chicken faeces and undergoes sporulation in the presence of humidity, warmth, and oxygen and thus becoming a sporulated oocyst, now infective (Lillehoj *et al.,* 2011). Merogony or schizogony occurs in the intestine and comprises of several rounds of asexual multiple division (from two to four times), followed by gametogony that involves the formation of male and female gametes, fertilization and formation of a zygote (oocyst) that will be excreted in faeces (Peek and Landman, 2011). Infection starts when the host ingest sporulated oocysts (Figure 2.1 and 2.2).

Due to the chemical and mechanical proventriculus and gizzard action, and the presence of CO_2 in the lumen, the oocyst releases the sporozoites, which gets into the intestinal lumen and attached to enterocytes using this anchoring and penetration proteins present in the apical complex (rhoptries and micronemes) entering the cell in order to continue the second phase: schizogony or merogony, producing a schizont with thousands of merozoites inside to be released back into the lumen to infect new intestinal cells (Lillehoj *et al.,* 2011). Thus, after several stages of merogonies, some of the merozoites inside the intestinal cell forms macrogamonts with a macrogametocyte (inmobile cell considered the female gamete) and some forms microgamonts with several microgametocytes inside (mobile cells considered male gametes) (Lillehoj *et al.,* 2011).These microgametocytes come out of the cells that originated to locate and fertilize to macrogametocyte producing a zygote to be excreted in the faeces again (not sporulated oocyst) to begin another cycle (Schito and Barta, 1997).

Once the oocyst is formed, it is considered the most persistent structure of *Eimeria* life cycle. It has a significant resistance to mechanical, chemical, and proteolytic damage, due to the composition of the two walls that confer the oocyst an outstanding resistance (Wallach, 2010; Lillehoj *et al.,* 2011).

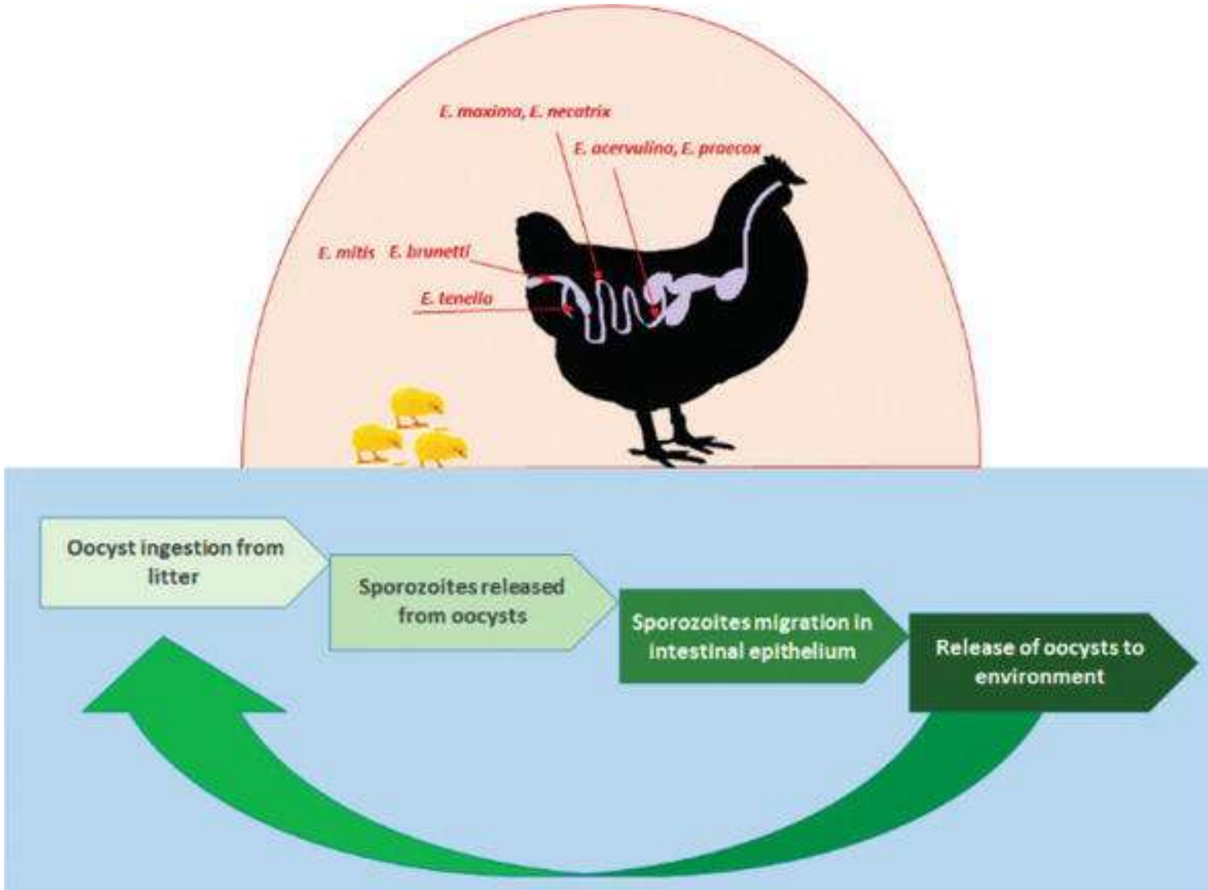

Figure 2.1: Infection process of *Eimeria* spp. in chickens. First, *Eimeria* oocysts are ingested by chickens, once inside, *Eimeria* sporozoites colonize and infect different regions of intestine epithelium. Oocysts are formed again and released to litter to begin another cycle.Source: https://doi.org/10.1016/j.trivac.2016.02.001.

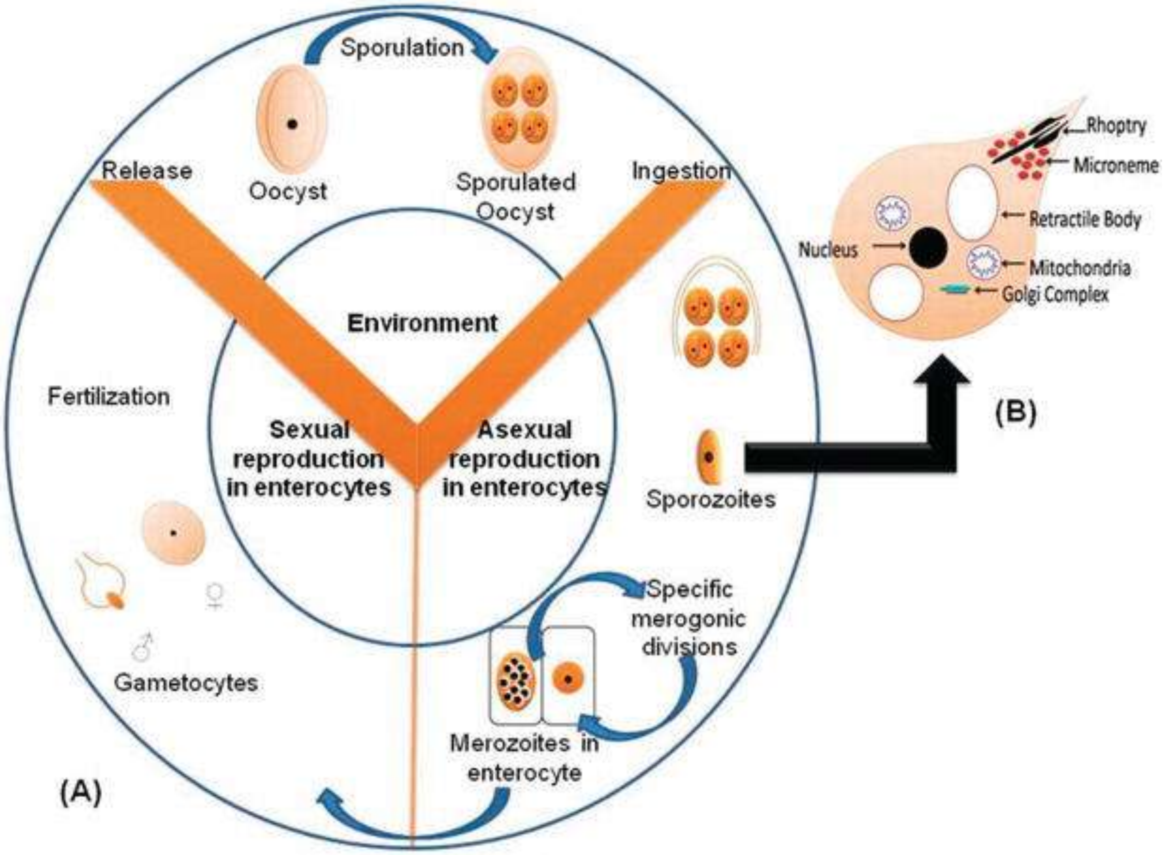

Figure 2.2: Life cycle of *Eimeria*. (A) Diagrammatic life cycle of *Eimeria* and (B) simplified structure of *Eimeria* spp. sporozoite. Source: https://doi.org/10.1016/j.trivac.2016.02.001.

2.1.10 Host cell invasion by *Eimeria* parasite

Apicomplexans, including all species of *Eimeria*, are highly successful obligate intracellular parasites. Unlike many microorganisms that rely on host-cellular pathways such as phagocytosis or pinocytosis for invasion, apicomplexans invade host cells rapidly and forcefully in a highly regulated, parasite-driven process (Santos *et al*., 2011).

Initial interaction with host cells can occur with the parasite in any orientation but commitment to invasion requires that the apical pole of the parasite makes irreversible contact with the host cell surface (Besteiro *et al*., 2011). This initiates formation of a moving junction (MJ), a tight focus of constriction between the parasite and host cell membrane, which migrates towards the posterior end of the parasite as invasion proceeds (Besteiro *et al*., 2011).

The parasite synthesizes specialized molecular complexes at the parasite–host interface, which are essential for gliding movement (the actinomyosin-dependent glideosome) and formation of the MJ (Lal *et al*., 2009). These complexes are assembled by regulated secretion of microneme (MIC) and rhoptry (RON/ROP) proteins, and interact with the parasite motor, localized in the pellicle, and with specific receptors on the host cell surface (Oakes *et al*., 2013). As the parasite propels itself forwards, surface-bound adhesion complexes are released by proteolysis. Other proteins, derived largely from the rhoptries, are secreted into the host cell where they contribute to the formation of a parasitophorous vacuole and its associated membrane, and modify the host intracellular environment (Boothroyd and Dubremetz, 2008).

The MJ was visualized many years ago (Aikawa, 1978) and is a key structure that provides an anchor, against which the parasite can generate a force that allows forward movement into the parasitophorous vacuole. A key component is apical membrane antigen-1(AMA1), a transmembrane protein secreted by the micronemes onto the parasite surface, where it interacts with secreted RONs to initiate formation of the MJ (Alexander *et al*., 2005; Lebrun *et al*., 2005). Collaboration between proteins that are secreted from different sub-cellular organelles requires a remarkable degree of orchestration (Alexander *et al*., 2005).

It has emerged recently that while AMA1 remains anchored on the parasite surface, a RON complex is secreted into the host cell and then one of them, RON2, becomes inserted into the host cell membrane and interacts directly with AMA1 (Tyler and Boothroyd, 2011). Both AMA1 and the RON protein repertoire are conserved in *Eimeria* indicating that the mechanism for forming the MJ is likely to be conserved in different coccidia (Blake *et al*., 2011a,b; Jiang *et al*., 2012; Lal *et al*., 2009; Oakes *et al*., 2013). It is worthy of note, however, that *E. tenella* expresses stage-specific variants of AMA1 and several of the RON proteins, which suggests that each zoite stage assembles a different set of gene products with which to build the MJ (Lal *et al*., 2009; Oakes *et al*., 2013).

2.1.11 Pathogenesis of coccidiosis

Infection is through ingestion of sporulated oocysts by oral route, with contaminated feed and/or water (Chapman *et al*., 2002; Nematullahi *et al.,* 2008). After ingestion, infectious oocysts excyst, liberating the infective form called the sporozoite. Sporozoites infect epithelial cells of the intestine. Transfer of the sporozoites up to the locus of the primary lesion is with the help of intraepithelial lymphocytes (Daszak, 1999).

The parasites invade the lining of the intestine and cause tissue damage, lowered feed intake, poor absorption of nutrients from the feed, dehydration, and blood loss (Fabiyi, 2000). The tissue damage can also expose the bird to bacterial infections, like *Clostridium* and *Salmonella*(Fabiyi, 2000). Diseases that suppress the bird's immune system may act with coccidiosis to produce a more severe problem. For example, Marek's disease may interfere with the development of coccidiosis immunity and infectious bursal disease may exacerbate a coccidia infection (Julie, 1999).

Pathogenesis of the infection is influenced by species of the coccidium, concurrent diseases and nutritional factors(Ferguson, 2003). *E. necatrix* and *E. tenella* are the most pathogenic in chickens because schizogony occurs in the lamina propria and crypts of epithelium of the small intestine and caeca, respectively, and causes extensive haemorrhage. Most species develop in epithelial cells lining the villi (Kahn, 2008).

The onset of mortality in a flock is rapid and mostly occurs between 5 and 6 days post infection (Ferguson, 2003). In acute infections, it may follow the first signs of infection by only a few hours, where the exact cause of death is not known, but toxic factors are suspected and in few cases, death may result from gangrenous or ruptured caecal pouches (McDougald and Reid, 1997).

2.1.12 Immunity to coccidiosis

Work by Rose and colleagues established the basic principles of immunity resulting from infection with *Eimeria*such as specificity, in which one species of *Eimeria*elicits little protection in birds against challenge with others (Rose and Long, 1962). Cell-mediated immune responses have been shown to be most important, humoral immunity playing little role in protection (Rose and Hesketh, 1979). Many types of cells of the immune system, including macrophages, dendritic cells, mast cells, natural killer cells, basophils, and eosinophils, have been shown to be involved in the innate immune response (Rose, 1996). The cytokine IFN-γ is found to be important in immunoregulation (Rose *et al.,* 1991), and subsequently, numerous cytokines and chemokines have been shown to be involved in primary and secondary responses to infection (Hong *et al.,* 2006; Gadde *et al.,* 2013). Recently evidence has been obtained that Toll-like receptors (TLR4 and TLR15) play a role in the innate immune response in *Eimerian* infections (Zhou *et al.,* 2013). Immunological variation between strains of the same species was first reported by Joyner for *E. acervulina* (Joyner, 1969) and subsequently shown to occur in *E.maxima*(Norton and Hein, 1976). These were the first of several studies showing that inherent intra specific variation could occur in biological characteristics of *Eimeria*(Jeffers, 1978). Several genotypic tools, including random amplification of polymorphic DNA-PCR, amplified fragment length polymorphism (RFLP), and gross chromosomal size polymorphism as revealed by pulsed-field gel electrophoresis, have recently been used to define inter and intra specific variation (Chapman *et al.,* 2013). Immunological variation between strains has been used by Blake and colleagues to map loci that encode strain-specific immunoprotective antigens (Blake *et al.,* 2011). From a practical perspective, the importance of immunological variation is that it could confound the use of vaccines because they might not confer protection against all strains of a species in the field. Recent transcriptome, proteome, and genome investigations by Tomley and colleagues (Lal *et al.,* 2009) have identified many antigens on the surface of sporozoites, some of which may suppress cell-mediated immunity and also contribute to proinflammatory responses and the associated pathology seen during infection (Lal *et al.,* 2009).

2.1.13 Clinical signs of poultry coccidiosis

Poultry coccidiosis affects birds in both clinical and sub-clinical forms. The clinical form of the disease manifests through prominent signs of mortality, morbidity, diarrhoea or bloody faeces, dehydration, lowered feed intake, weight loss,paleness, huddling, ruffled feathers, and depression (Julie, 1999; Taylor *et al*., 2007; Muazu *et al.*, 2008; Ali *et al.*, 2014). The chicken affected with coccidia become tired and weak, young birds often have blood in the faces after 4-5 days, later their eyes are closed and their wings hang down and many birds die (Julie, 1999; Yu-wen, 2009).

The occurrence of clinical coccidiosis is directly related to the number of sporulated oocysts ingested by a bird at one time, the pathogenicity of the *Eimeria* species (studies carried outin Zariaindicates*E. tenella*and *E. necatrix* to be the most pathogenic), the age of the infected chicken and the management system (Musa *et al.,* 2010). Chickens suffering from coccidiosis quickly become less productive and poor performers. Laying hens will experience a reduction in rate of egg production (Nematullahi *et al*., 2009). The sub-clinical coccidiosis manifests mainly by poor weight gain and reduced efficiency of feed conversion and gives rise to highest proportion of the total economic loss (Taylor *et a*l., 2007).

2.1.14 Gross lesions of poultry coccidiosis

The lesions of coccidiosis depend on the degree of inflammation and damage to the intestinal tract (McDougald and Hu, 2001). They include thickness of the intestinal wall, mucoid to blood-tinged exudates, petechial haemorrages, necrosis, hemorrhagic enteritis and mucous profuse bleeding in the ceca (McDougald and Hu, 2001).

Coccidiosis causes a thickening of the intestines which make them feel like sausage(Fanatico, 2006). There may be light coloured spots on the surface of the gut, and inside the gut haemorrhages and streaks. The type and locations of lesions in the gut indicates the species of genus *Eimeria* (Fanatico, 2006) as explained in Table 2.1.

2.1.15 Histopathological lesions of poultry coccidiosis

Histopathological examinations of of the caeca revealedtissue damage, sloughing of theepithelial cells associated with the infiltration of eosinophils,heterophils and lymphocytes in the lamina propria, presence of clusters of large schizonts and merozoites in the caecal tissue, and coccidia oocysts in the lumen (Marquardt *et al*., 2000; Ferguson *et al*., 2003). The wall of the gut is thickened indicating retention of fluid (edema). There may be blood in the lumen of the gut indicating blood loss (haemorrhage), or merely retention of an excessive amount of blood in the tissue (hyperemia),as reported by Adamu and Chaiwat (2013).

2.1.16 Diagnoses of poultry coccidiosis

2.1.16.1 Bases of Diagnosis

Diagnosis of coccidiosis in poultry flocks is based on history,clinical signs, coprology and pathomorphological and pathohistological analysis (Conway & Mckenzie, 2007). Nowadays, various biochemical and molecular methods have also been used (Morris & Gasser, 2006). Clinical findings like the presence of faeces with blood dysentery and diarrhoea suggests coccidiosis (Vegad, 2004). Serology is the predominant method of disease monitoring in commercial poultry; examination of blood smears, bone marrow and clinical chemistry values is rarely done (Wakenell, 2010).

Table 2.1: Some characteristic lesions of *Eimeria* infection in poultry during post mortem examination

Eimeria species	Location	Lesion
E. necatrix	Middle intestine	Severe haemorrhage with mucoid discharge whitish and red spot in wall of intestine
E. maxima	Middle intestine	Distended intestine with haemorrhage spots, mucoid discharge
E. brunetti	Lower half of intestine	Thin walled intestine, mucoid on necrotic discharge, distension of intestine
E. tenella	Caeca	Severe haemorrage with white red spots in wall of intestine
E. acervulina	Upper intestine	Whitish spots on wall on serous surface haemorrage streak and whitish lesions on intestinal surface, mucoid enteritis
E. praecox	Duodenum	No lesion but slightly haemorrhagic appearance on intestinal surface of duodenum slight mucoid discharge

Source: Saxema *et al*. (1998)

2.1.16.2 Detection of oocyst in faeces

Flotation method with saturated salt or sugar solution is important for the detection of oocysts in faeces of infected birds (Conway & Mckenzie, 2007). It can be a useful indicator of subclinical infection (Arabkhazaeli *et al*., 2011). Concentration flotation technique is also used for the collection of *Eimeria* oocysts from intestinal content of chickens. *Eimeria* oocysts isolation depends on the measurements of oocysts by using a calibrated ocular micrometer at 400x magnification (Conway & Mckenzie, 2007). Oocyst size, shape, and minimum sporulation per time are helpful in identification of *Eimeria* species (Arabkhazaeli *et al*., 2011).

2.1.16.3 Examination of gross lesions

Diagnosis of coccidiosis in chicken is best accomplished by post mortem examination of a representative number of birds (Hadipour *et al*., 2011). The characteristics of the observed lesions such as its location on the intestinal tract, its appearance and severity, the nature of intestinal contents like mucoid to blood-tinged exudates, petechial haemorrhages, can be useful in establishing a diagnosis (Conway and Mckenzie, 2007).

2.1.16.4 Microscopic examination of lesions

Developmental stages of coccidia can be seen in smears taken from suspected mucosal lesion. Oocysts, schizonts or gametocysts are easily seen and can be pathogenic for certain species of coccidia. Theinformation from microscopic examination should be combined with other observations such as gross lesions and identification of individual species (Mcdougald and Reid, 1997).

2.1.16.4 Molecular diagnosis

During recent years, there have been significant advances in the development of moleculardiagnostic tools (Lien *et al*., 2007). Molecular techniques have some advantages over traditional methods in that they rely only on the genomic sequence of the *Eimeria* species. Several Polymerase Chain Reaction (PCR) based assays using primers that are specifically targeting different regions of the *Eimeria* genome have been described (Ogedengbe *et al*., 2011).

2.1.17 Differential diagnoses of coccidiosis

Intestinal coccidiosis may be confused with necrotic and ulcerative enteritis, haemorrhagic enteritis, ascariasis, capillariasis, salt poisoning, mycotoxicoses or other enteric diseases. Caecal coccidiosis may be mistaken for Newcastle disease, histomoniasis (blackhead), and salmonellosis due to their similar lesions (Hafez, 1997). Other conditions include: cannibalism, intestinal torsion and intussusception (Abdu and Musa, 2019).

2.2 Control of Poultry Coccidiosis

2.2.1 Chemotherapy

The effective use of anticoccidial feed additives over the past 50 years has played a major role in the growth of the poultry industry and has allowed the increased availability of highquality, affordable poultry products to the consumer. These anticoccidials can be classified as (i) chemicals which have specific modes of action against parasite metabolism, such as amprolium, clopidol decoquinate, halofuginone, or (ii) polyether ionophores such as mechanisms of altering ion transport and disrupting osmotoic balance (Jeffers, 1997). These latter compounds are now the mainstay of coccidiosis control (Jeffers, 1997). A compendium of the most frequently used anticoccidials is available through Janssen Pharmaceutica (Fowler, 1995). It is quite clear, however, that some degree of resistance to all anticoccidial drugs, including ionophores, has developed(Chapman, 1994, 1998; Martin *et al*., 1997). To minimize the effects of resistance, poultry producers rotate the use of various anticoccidials with successive flocks, combine chemical and ionophore treatments, or employ shuttle programs during a flock grow out (Williams, 1998). Application of these treatment programs depends on seasonal conditions and prevalence of various species of coccidia (Williams, 1998). In recent years, pharmaceutical companies have not brought new anticoccidials to market. However, two potential drug targets, enzymes of the sporozoite mannitol cycle (Schmatz, 1997; Allocco *et al*., 1999) and trophozoite histone deacetylase, have been identified (Schmatz, 1997).

2.2.2 Vaccination

2.2.2.1 Vaccine development

Avian coccidia are highly immunogenic, and primary infections can stimulate solid immunity to homologous challenges (Lillehoj *et al*., 2000). Therefore, it would seem obvious that vaccines could offer excellent alternatives to drugs as a means of controlling coccidiosis. Efforts to develop various types of vaccines (Lillehoj and Trout, 1993; Lillehoj and Lillehoj, 2000; Lillehoj *et al*., 2000) have been continuous over the past several decades, but progress has been slow. Highlights of research on vaccine development include:

2.2.2.2 Recombinant vaccines

Much research effort has been spent on the development of recombinant vaccines. Although none are in commercial use, this research has served to highlight the complex nature of the avian host-coccidia interaction. A major hurdle to overcome in the development of a recombinant vaccine is the lack of crossspecies immune protection (Jenkins, 1998; Vermeulen, 1998). Other factors impeding the development of a successful vaccine have been recently reviewed (Jenkins, 1998; Vermeulen, 1998), the most important of which is the identification of protective antigens. Many potential coccidial antigens have been characterized and cloned. A total of 29 DNA sequences encoding immunity-stimulating *Eimeria* proteins from various species and developmental stages have been listed in a review (Jenkins, 1998). Many of these antigens are surface proteins or internal antigens associated with organelles such as micronemes (Tomley *et al*., 1991; 1996), rhoptries (Tomley, 1994), and refractile bodies (Vermeulen *et al*., 1993). Tennyson and Barta(2000) identified 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 (Jenkins *et al*., 1991a, 1991b, 1993)), merozoite antigens (Brake *et al*., 1997; Vermeulen, 1998), and gamete antigens (Wallach *et al*., 1995) all elicit various degrees of protective immunity. A delivery mechanism for coccidial vaccines that produces optimum resistance to challenge infection has yet to be determined. Immunogenic *Eimeria* antigens have been administered as isolated proteins with adjuvants (Brake *et al*., 1997; Vermeulen, 1998), as recombinant antigens in live vectors such as nonpathogenic strains of *Escherichia coli, Salmonella enterica* serovar *Typhimurium*, poxviruses, fowlpox virus, and turkey herpesvirus (Tomley *et al*., 1991), and by direct plasmid DNA injection (Lillehoj*et al*., 1997; Jenkins *et al*., 1998; Kopko *et al*., 2000), with various degrees of success. More research is needed to determine how to target vaccines to the cellular immune effector systems that operate during natural primary and challenge infections and to determine the unique host immune responses to each parasite. Because T-cell-stimulatory antigens appear to be the most relevant to protective immunity, T-cell proliferation assays (using T cells from recovering chickens) and IFN- production have been used to screen for protective antigens against *E. tenella* infections (Breed *et al*., 1997). The fraction of antigens that producedthe highest T-cell proliferation and macrophage-activating activity, when administered as a vaccine, also yielded chickens with the lowest caecal lesion scores after challenge (Breed *et al*., 1997).

2.2.2.3 Live vaccines

Live vaccines for coccidiosis control have been used to a limited degree by the poultry industry for about 50 years. Their effectiveness hinges on the recycling of initially very low doses of oocyst, and the gradual buildup of solid immunity. They have been used primarily to protect breeder and layer flocks (Shirley *et al*., 1995). However, their use, particularly in broiler flocks, is increasing(Shirley *et al*., 1995). Live vaccines contain either attenuated or virulent coccidial strains. Paracox (consisting of precocious strains of all seven species of chicken coccidia) and Livacox (consisting of precocious and egg-passaged lines) are attenuated vaccines (Bedrnik *et al*., 1995). All coccidial strains in these vaccines are drug sensitive(Bedrnik *et al*., 1995). Coccivac and Immucox are each composed of several virulent species (Bedrnik *et al*., 1995). All four vaccines are commercially available. An in-depth comparison of these vaccines is provided by Bedrnik *et al*. (1995). All of these vaccines are administered during the first week after hatch and will produce solid immunity when they are used carefully under good rearing conditions (Danforth, 1998). Because live vaccines are multivalent (containing more than one species), tests of their efficacies require a different approach from tests of the efficacies of anticoccidial compounds. A standard protocol that is based on growth rates after challenge and that avoids lesion scoring and oocyst output enumeration has been published (Williams and Catchpole, 2000). An advantage of attenuated vaccines is that they have 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 (Williams, 1994). It is believed that the drug-sensitive, attenuated strains and wild, native strains interbreed, reducing both virulence and drug resistance in local populations. Thus, the useful period of anticoccidial drugs could be extended by rotating their applications with live vaccines (Williams, 1998). Coccivac and Immucox have also been used to treat broiler breeders, particularly in the United States and Canada (Allen and Fetterer, 2002). There has been a general reluctance to try them in broilers and heavy roaster birds, because of reduced weight gain and feed conversion compared to those in prophylactically medicated birds (Danforth, 1998). Additionally, there is a risk of introducing unwanted *Eimeria* species into the environment. However, battery, floor pen, and broiler house trials (Danforth, 1998) have shown that this type of vaccine (e.g., Immucox) can provide equal or superior performance, compared with prophylactic medication, when given in gel form at 1 day of age, because this method ensures the synchronous exposure of all birds to small uniform numbers of oocysts (Danforth *et al*., 1997a, 1997b). Antigenicity of coccidial strains can vary geographically (Fitz-Coy, 1992; Martin *et al*., 1997). Therefore, it is important to screen live vaccines against local poultry populations before administering them to large flocks. Immunovariant strains can be isolated and substituted to provide region-specific, autogenous vaccines (Danforth, 1998). Live vaccines can also be modified by incorporating drug-resistant strains. In floor pen trials, anticoccidially medicated broiler birds that had been immunized at 1 day of age with a drugresistant strain of coccidia were protected almost completely against homologous challenge (Danforth, 1998). When this vaccination regimen was applied to large flocks in two sets of paired broiler houses, vaccinated-medicated birds had consistently lower (more efficient) feed conversions but slightly more variable final weights than did the medicated controls (Danforth, 1998). Thus, this type of vaccine treatment has the potential to extend the life of anticoccidials cleared for use in geographical areas where drug resistance and lack of coccidial control is a problem (Danforth, 1998).

2.2.2.4 Use of cytokines as adjuvants

Cytokines are major modulators of immune responses to infection, and therefore they represent natural sources of immunostimulation that could be used as adjuvants in vaccines. IFN- has received the most attention as a possible adjuvant for an anticoccidial vaccine. Chicken IFN- has been cloned (Digby and Lowenthal, 1995; Lowenthal *et al*., 1995; Song *et al*., 1997). Treatment with recombinant IFN- alone has been shown to moderate reductions in weight gain (Lowenthal *et al*., 1997; Lillehoj and Choi, 1998) during *E. acervulina* and *E. tenella* infections and to moderate oocyst output (Lillehoj and Choi,

1998) during *E. acervulina* infection. One problem with the *in vivo* use of cytokines is their rapid degradation and clearance (Lowenthal *et al*., 1999). Administering them in DNA form or in a viral or bacterial vector could overcome this problem and provide a more practical method for treating large flocks. For example, a recombinant protein (3-1E) from *E. acervulina* merozoites, which is also found in *E. tenella* sporozoites and merozoites, stimulated IFN- production in chicken spleen lymphocytes. Direct injection of 3-1E cDNA induced protective immunity against *E. acervulina* challenge. Simultaneous injection of cDNA encoding chicken IFN- further enhanced immunity (Lillehoj *et al*., 2000). Chickens treated with a fowl adenovirus chicken IFN- construct showed increased weight gains compared to untreated controls and exhibited smaller weight gain reductions on challenge with *E. acervulina* (Johnson *et al*., 2000).

2.3 Economic Impact of Coccidiosis

Chickens are economically significant poultry species due to their high protein content, short generation interval, and global acceptability (Blevin *et al.,* 2018; Qi *et al.,* 2018). However, the subclinical and clinical effects of coccidiosis result in malabsorption, reduced feed conversion rate and weight gain, and an increase in mortality and susceptibility to secondary disease in infected chickens (Prakashbabu *et al.,* 2017). An outbreak of coccidiosis has a very high negative and economic impact on the flock as well as for the poultry producer as treatment alone cannot prevent the economic losses (Majaro, 1980; Barkash, 1989). The disease results in overall 51.38% mortality in the poultry industry worldwide (Cocciforum, 2007). Consequently, the livestock industry suffers a global loss exceeding $3 billion dollars, making precautions to control the disease fundamental (Cheng *et al.,* 2018). In Nigeria where poultry farming is less developed, the disease is more serious and causes heavy economic losses. The losses associated with coccidiosis include both direct and indirect components; including the cost of control measures, production losses and potential consequences to animal health from resistance to chemoprophylaxis could be in the region of millions of Naira (Maikai *et al.,* 2007; Fornace *et al.,* 2013). The disease therefore carries losses for the farmer in the form of mortalities, reduced market value of the affected birds and sometimes culling or delayed slaughter time (Yu-Wen, 2009).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Location

The study was carried out at the Poultry Research Pens of the Veterinary Teaching Hospital, Ahmadu Bello University, Zaria. Zaria is located in the Northern Guinea savannah, between Latitude 11^{o} and 12^{o}N and Longitude 7° and 8^{o}E standing at an elevation of 650 metres above sea level (Sawa and Buhari, 2011). It has a tropical continental climate with a pronounced dry season, lasting up to six months (November - April). The average rainfall ranges from 1,000 – 1,250 mm and the average daily temperature ranges from 19 ^{0}C – 33 ^{0}C (Sawa and Buhari, 2011).

3.2 Period of the Study

The study was conducted during the months of October and November, 2019.

3.3 Study Design

The study design was an experimental study.

3.4 Ethical Clearance

Ethical clearance to conduct the study was sought and obtained from the Ahmadu Bello University Committee on Animal Use and Care (ABUCAUC) with approval number ABUCAUC/2021/001 (Appendix 27).

3.5 Housing and Feeding

Two of the Poultry Research Pens containing four cages were cleaned, washed with water and detergent, and disinfected with Diskol® (5% benzalkonium chloride, 7.5% glutaraldehyde, 7.5% formaldehyde, 9.5% stabilizers and antioxidants) at the rate of 40 ml/10L of water, sodium hypochloride (Bleach®) and 10% formalin seven days before the experimental chicks arrived. Each cage measuring 600 mm × 500 mm × 430 mm contained 20 birds. The cages were wire-floored batteries and raised to a height of 10 cm from the floor of the pen toallow passage of faecal droppings. Electric bulbs and large netted wire-meshed windows were used to maintain the recommended temperature, light; and control against insects/rodents within the pens. The birds were fed with a coccidiostat-free feed and clean tap water *ad libitum*using galvanized feeders and plastic drinkers respectively.

3.6 Experimental Chicks

One hundred day-old Arbor Acre broiler chicks were purchased from a hatchery in Ibadan, Nigeria. On arrival the broiler chicks were given glucose and multivitamins in drinking water. The chicks were brooded in two of the four cages used in the experiment, which were properly cleaned and disinfected before their arrival. Newspapers served as litter material. The chicks were raised together for 2 weeks and screened every other day for *Eimeria*infection by microscopic examination of the pooled faeces for *Eimeria* oocyst prior to grouping, vaccination and subsequent inoculation.

3.7 Experimental Design

Eighty two-week-old Arbor Acre broiler chickens were randomly divided into four groups, after they have been brooded together for two weeks, with each group consisting of 20 birds.The groups were: G1, the unvaccinated, unchallenged (negative control) group; G2, unvaccinated, challenged with *E. tenella*local isolate (positive control) group; G3, the vaccinated, challenged with *E. tenella*local isolate group and the G4, vaccinated, challenged with *E. tenella* Houghton strain. G3 and G4 wereimmunized with 100 µg EtAMA1 DNA vaccine intramuscularly (i.m.) in the leg muscleon day 14 and a booster vaccination at day 28 of age.Seven (7) days after the booster vaccination,chickens in G2 and G3 were inoculated orally with 2×10^4 sporulated oocysts of *E. tenella* local isolate while those in G4 received2×10^4 sporulated oocysts of *E. tenella* Houghton strain of the parasiteusing an insulin syringe. This was achieved by opening the beakof each chick and depositing the inoculum directly into the mouth one after the other through manual restraint. The sporulated oocysts suspended in distilled water were agitated each time before drawing with the syringe.All chicks were monitoreddaily for 7 days post infection for clinical signsof caecal coccidiosis and weighed individually in each group, using a weighing balance on day 0 before inoculating with the sporulated oocysts and on day 7 post infection. These values were used to determine the mean body weight gain of each group by subtracting the body weight of the birds at day 0 pre-infection from the body weight of the chickens on day 7 post inoculation.Feed conversion ratio was obtained for each group of birds by dividing the total weight of feed consumed (g) by the weight gain (final weight – starting weight) (g). Feed consumption was assessed based on daily feed intake (DFI) of birds per group from day 0 pre-infection to day 7 post infection by subtractingquantity of feed left from quantity of feed offered per day Survival rate was determined according to groups by dividingthe number of surviving chickens by the number of initial chickens at the time of challenge multiplied by one hundred. Blood samples were collected by venipuncture from five birds in each group on day 14, 21, 28 and 35 of age to analyse for haematological parameters, total plasma protein and the level of serum immunoglobulin and cytokine. The caeca were harvested on day 35 of age (7 days post-infection) at postmortem to examine for gross and microscopic lesions consistent with coccidiosis(Table 3.2).

3.8 Vaccine

3.8.1 Type and source of the vaccine

3.7.1.1 Type of vaccine

The vaccine used in the study was a recombinant vaccine of *Eimeria tenella* apical membrane antigen-1 (EtAMA1) construct (Blake *et al*., 2017).

3.7.1.2 Source of vaccine

The DNA vaccine was provided by Damer P. Blake, a Professor of parasite genetics from the Department of Pathology and Pathogen Biology of the Royal Veterinary College, University of London, UK. It was received as a bacterial stock cultured on an agar slope containing the plasmid DNA encoding the *Eimeria tenella* apical membrane antigen-1

(AMA1) in a pcDNA3.1 DNA vaccine vector, *Escherichia coli* XL1 Blue strain sealed in a polythene bag kept under room temperature.

3.8.2 Propagation and purification of the plasmid DNA

3.8.2.1 Propagation of the plasmid DNA

The bacterial stock wastaken to the Biotechnology Laboratory of the Molecular Bacteriological Unit, National Veterinary Research Institute, Vom. It was grown in a Standard Lysogeny broth (LB) supplemented with ampicillin as a selective factor for propagation of the antigen.

3.8.2.2 Purification of the plasmid DNA

The plasmid DNA was extracted (purified) from the DNA vaccine vector using Zyppy™ Plasmid Miniprep Kit according to the manufacturer's instructions to produce the needed quantity of DNA for vaccination of the birds.

3.8.3 Expression of the gene (EtAMA1) in the plasmid DNA

Two primers (oligonucleotides) that targeted a mid-section of the *E. tenella* AMA1 gene were used to confirm its presence in the recombinant DNA. The primers used were forward5′ATAGGATCCGAGCTGCGCAGGGCCGGCAGCA3′ and reverse 3′GCGAAGCTTTTAACCGCCCCCTTTAGACTCGC5′.

3.9 Immunoglobulin Y (IgY) and Interferon Gamma (IFN-γ) ELISA Kits

An indirect enzyme linked immunosorbent assay (ELISA) of the Chicken Immunoglobulin Y (IgY) and Chicken Interferon Gamma (IFN-γ) ELISA Kits of Abbkine, Inc – China in duplicate were purchased to measure the concentrations of immunoglobulin (IgY) and interferon gamma (IFN-γ) respectively in serum of the chickens in each group.

3.10 Sources of the *Eimeria tenella* Oocysts

3.9.1 Local isolate of *E. tenella*

Theseoocysts were obtained from the Department of Veterinary Parasitology and Entomology, Ahmadu Bello University Zaria. Thecoccidia was previously isolated and characterized by Jatau *et al*. (2016). Potassium dichromate solution (2.5%) was used to store the sporulated oocysts. The solution was washed off by centrifuging at 2000 rpm for 12 minutes in test-tubes to sediment the sporulated oocysts to the bottom of the test-tubes and the supernatant was then decanted (Collee *et al*., 1982). The procedure was repeated three times and the sediment containing the oocysts were pooled together. Adequate number of sporulated *E. tenella* oocysts for experimental inoculation were obtained by propagating the presevered oocysts(Jatau *et al*., 2016), in five coccidia-free chickens, which were monitored until bloodydiarrhoea and other signs of caecal coccidiosis were observed. The caeca of the five infected birds were harvested, the oocysts contained in them pooled together and sporulated for use in the experiment.

3.10.2 Houghton strain of *E. tenella*

The Houghton (H) strain of *Eimeria tenella*oocysts was provided by Damer P. Blake, a Professor of parasite genetics from the Royal Veterinary College, University of London, UK. The H strain was isolated in 1949 in the UK and was maintained initially at the

Houghton Poultry Research Station (HPRS), Houghton, and thereafter at theInstitute for Animal Health (IAH), Compton, UK following the closure of the HPRS laboratory in 1992. It is one of the most widely studied strains. At various times the parasite has been provided to other institutions and research groups carrying out coccidiosis research in the fowl, and utilised in many fundamental studies on the Eimerian life cycle and its relationship with the host (Chapman and Shirley, 2003).

3.11 Sporulation of *Eimeria tenella* Oocysts

A saturated sugar solution with a specific gravity of 1.3 (Collee *et al*., 1982), wasprepared and used as a flotation medium. Faeces with fresh saturated sugar solution were mixed thoroughly using a pestle to homogenize the mixture. The faeces and saturated sugar solution were sieved through a tea strainer into a beaker to remove faecal debris. The oocysts were then allowed to float in the saturated sugar solution for about 20 minutes. 1 ml of the filtrate was conveyed from the surface into a testtube using a pipette. This was then mixed with 9 ml of distilled water and centrifuged at 2000 rpm for 12 minutes to sediment the oocyst to the bottom of the test-tube.The supernatant was discarded using a dropper and replaced with tap water (Collee *et al*., 1982). Washing was repeated three times and the sediment containing the oocysts were poured into petri dishes containing 2.5% potassium dichromate solution as a thin layer and were allowed to sporulate for one week at room temperature (28^{o}C) (Venkateswara*et al*., 2013). The filtrate was kept in wide mouth containers with constant aeration to allow 90 per cent sporulation of oocysts (Venkateswara*et al*., 2013). Evaporated potassium dichromate from the mixture was also constantly replaced by addition of the dichromate solution. The oocysts were checked daily by homogenizing the dichromate solution, placing a drop of it on a glass slide and viewing under a light microscope for sporulation (Venkateswara*et al*., 2013). After one week the potassium dichromate solution was washed off by centrifuging at 2000 rpm for 10 minutes. This was repeated three times. After the final wash, the cleaned oocysts were re-suspend in a suitable volume of water andenumerated using McMaster counting technique as described by Rose and Mockett (1983). The solution was then decanted and fresh potassium dichromate was added to the oocysts and stored at 4 ^{o}C until it was used to infect the birds.

3.12 Preparation of Inoculum

The number of oocysts per ml of the inoculum was determined using the McMaster technique. This was achieved by washing off the potassium dichromate solution used to preserve the sporulated oocysts by centrifugation to sediment the oocysts, the washed sporulated oocysts were re-suspended in distilled water; giving an estimated number of 100,000 oocysts per ml of the inoculum (Rose and Mockett, 1983).

3.13 Observation for Clinical Signs

Infected chicks were monitored individuallydaily for 7 days post infection for clinical signswhich were presented in percentage, on a scale of 1 to 5, and graded as mild, moderate,severe, very severe and grave (Kilyobas, 2018) (Table 3.1).

3.14 Determination of Body Weight Gain

The birds were weighed individually in each group, using a weighing balance on day 0 before inoculating with the sporulated oocysts and on day 7 post infection. These values were used to determine the mean body weight gain of each group by subtracting the body weight of the birds at day 0 pre-infection from the body weight of the birds on day 7 post inoculation. Thus, body weight gain (BWG) =

Final body weight (7 days post inoculation) (g) – Initial body weight (0 day post inoculation) (g)

3.15 Assessment of Feed Conversion Ratio

Feed conversion ratio was obtained for each group of birds by dividing the total weight of feed consumed (g) by the weight gain (final weight – starting weight) (g). Feed consumption was assessed based on daily feed intake (DFI) of birds per group from day 0 pre-infection to day 7 post infection by subtractingquantity of feed left from quantity of feed offered per day (Ayssiwede *et al*.,2011).

$$\text{Thus, feed conversion ratio (FCR)} = \frac{\text{Total feed consumed (g)}}{\text{Weight gain (final weight – initial weight) (g).}}$$

3.16 Determination of Survival Rate

Survival rate was determined according to groups by dividingthe number of surviving chickens by the number of initial chickens at the time of challenge multiplied by one hundred (Wang*et al*., 2020).

$$\text{Survival rate} = \frac{\text{Number of surviving chickens}}{\text{Number of initial chickens at the time of challenge}} \text{x100}$$

3.17 Determination of Oocysts Count

Oocysts count per gram of faeces was determined per bird using the McMaster's counting technique (Subramanian *et al*., 2008). Two grams of faeces were scooped into a centrifuge tube and mixed with 6 ml of saturated salt solution, then centrifuged at 2000 rpm for 12 minute after which the supernatant was decanted into a dropper bottle this made up the first harvest. Another 6 ml of saturated salt solution was poured into a centrifuging tube which contained the sediment. This was shaken and then centrifuged at 2000 rpm for 12 minutefor the second harvest of the oocysts. The supernatant was then decanted into a dropperbottle and shaken to make it to 12 ml. A chamber of the McMaster slide was then filled with the supernatant using a pipette. The suspension was mixed again and the second field of the slide was filled. The oocysts were allowed to float for at least 2 min and all the oocysts under the two separate grids of the McMaster slide were counted at ×10 objective. This was used to calculate the number of oocysts per gram of faeces.

Table 3.1: Clinical sign of caecal coccidiosis in percentage, on a scale of 1 to 5 and grade

Percentage (%)	Scale	Grade
01– 20	1	Mild
21– 40	2	Moderate
41– 60	3	Severe
61– 80	4	Very severe
81– 100	5	Grave

Source: Kilyobas, 2018

3.18 Determination of Oocyst Decrease Ratio

Oocysts per gram (OPG) of content was determined using the McMaster's counting technique (Subramanian *et al*., 2008). Oocyst decrease ratio (ODR) was calculated as:The mean number of oocysts from positive control chickens - vaccinated chickens/positive control chickens × 100. This is calculated as: ODR = (A – B) A × 100

Where A = mean number of oocysts in chickens infected and untreated.

B = mean number of oocysts in chicks treated with the extracts fractions.

3.19 Determination of Anti-coccidial Index

The anti-coccidial index (ACI) is a synthetic criterion for assessing the protective effect of a medicine or vaccine against coccidiosis, established by Merck Sharp & Dohme (1976), was calculated as ACI = (rBWG + survival rate) × 100 − (lesion score + oocyst value).

The ACI usually ranges from 0 – 200 (Jeffers, 1974;Chapman, 1998; Yegani and Korver, 2008). According to McManus*et al*. (1968),an ACI ≥180 is considered as highperformance, an ACI between 160 and 179 is considered effective, while value of ACI < 160 is considered ineffective.

3.20 Evaluation of Haematological Parameters

3.20.1 Determination of packed cell volume

The packed cell volume (PCV) per bird was determined using the microhaematocrit method (Feldmann *et al*., 2000).Microhaematocrit capillary tubes was filled with blood by capillary action to about 75%of their lengths; the outside of the tubes were carefully dried with cotton wool after filling each of the tubes with blood. The opposite ends were then sealed with a flame from a Bunsen burner; the sealed tubes were then placed in a microhaematocrit centrifuge with their sealed ends placed near the outside rim of the centrifuge touching the rim. The microhaematocrit centrifuge cover was then replaced and tightened. It was then turned on for 5 minutes at 10, 000 rpm. The spun tubes were now removed and placed on a microhaematocrit tube reader one after the other to determine the PCV in percentage.

3.20.2 Determination of haemoglobin concentration

Haemoglobin concentration was assayed colorimetrically as cyanomethhaemoglobin (Drabkin, 1945). Five millilitres of HICN (Drabkin) solution was measured per bird using a

5 ml syringe into plastic test tubes. Twenty microlitres (20 µl) of blood were measured using a micropipette and added to the Drabkin solution in the test tube and properly mixed by gently shaking the test tube. It was centrifuged at 1,509 x *g* for 15 minutes to separate the empty RBC from interfering with the reading. The supernatant was separated into a sample bottle. The mixture was absorbed into the haemoglobin meter (XF–C, China). After the wiggling pump stops working, the value displayed on the screen was recorded in g/dl as the haemoglobin concentration(Drabkin, 1945).

3.20.3 Determination of total red blood cell and white blood cell count

Red blood cells (RBC) and total WBC (or TWBC) counts were determined with the Natt-Herrick solution (1:200 dilution) and the Improved Neubauer haemocytometer (Campbell and Ellis, 2007) as both counts can be prepared directly from the same sample placed in the haemocytometer.

The heparinised blood samples were slightly agitated and the RBC diluting pipette was used to pipette the blood to the 0.5 marking. The tip of the pipette was cleaned properly using a tissue paper without touching the distal opening of the pipette tip with tissue, as this will cause capillary shift of blood into the tissue. The diluting solution (Natt-Herrick) was also pipetted to the 101 marking (1:200) without entirely immersing the pipette tip into the diluting fluid. The mixture was well shaken for 1 minute to obtain equal distribution then emptied into a clean sample bottle. The Neubauer haemocytometer and cover slip were cleaned using a dry, lint free cloth. The cover slip was properly placed on the haemocytometer. The mixture was then agitated a little and a capillary tube was used to withdraw a small aliquot. Both sides of the haemocytometer were filled up (charged) by gently touching the intersection between the cover slip and haemocytometer with the loaded capillary tube avoiding air bubbles and under-filling or over-filling, then left for 5 minutes for cells to settle down.

The light microscope (Olympus-XSZ-107BN), at low power magnification (x40) was used to view the cells and counting was done using the tally counter (Campbell and Ellis, 2007).

For TWBC count, the WBC in the four outer large squares of the haemocytometer were counted and calculated using the formula below:

$$N/20 = \text{WBC} \times 10^{9} /\text{L}$$

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

For RBC count, the cells contained in the four corner and central squares in the mid-section of the haemocytometer were counted. Following the "L" rule: cells that touch the centre triple lines of the ruling on the left and the bottom sides were counted but cells that touch the centre triple lines of the ruling on the right and the top sides were not counted (Samour, 2001). The RBC count was calculated using the formula below:

$$N/100 = \text{RBC} \times 10^{12} /\text{L}$$

Where N = Number of RBC counted in the 5 squares in the mid-section of the haemocytometer (or in 160 squares)

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

3.20.4 Determination of differential leukocyte count

In all the birds, a pair of smears for each blood sample was made. A small drop (about 2 µl) of blood was immediately used for the preparation of blood smears each using the standard slide-to-slide technique (Samour, 2001). The air-dried smears were properly labelled using a pencil on the frosted end of the slide and then fixed in a fixing jar containing methanol for 3 minutes and air-dried.

Staining was done by flooding the smears with Wright-Giemsa stain for 3 minutes. An equal amount of Sorensen's buffer (pH of 6.8) was added then mixed gently by blowing using a pipette until green metallic sheen forms on the surface. This was allowed to stand for further 6 minutes. The smears were rinsed with the Sorensen's buffer and allowed to stand for a minute for differentiation. The stained slides were then washed copiously with the Sorensen's buffer and the back of the smears were wiped with tissue paper to remove the excess stain and allowed to air dry. These were neatly packed into a slide box until viewing.

Examination of the blood smears was done using a light microscope (Olympus-XSZ-107BN) (Binocular Microscope - Biological & Chemical Laboratory, Pomona, California, United States) under high-power magnification with oil immersion (×1,000). One hundred WBC were counted and classified based on their morphologic features (Hawkey and Dennet, 1989; Campbell and Ellis, 2007). The counting was done using the Marble® Blood Cell Calculator. The differential WBC count was then expressed as a percentage of the individual cell group (Campbell and Ellis, 2007). The percentage of each cell was then converted into absolute numbers by reference to the total WBC using the formula below:

<u>Percentage of WBC counted x TWBC</u> = Absolute Number x 10^9 /l

100

3.20.5 Calculation of mean corpuscular volume and haemoglobin

The mean corpuscular volume (MCV) and mean corpuscular haemoglobin (MCH) were calculated as described by Coles *et al*. (1986);

$$MCV\ (fL) = \frac{PCV\ (\%) \times 10}{RBC\ (\times 10^{12}/L)}$$

$$MCH\ (pg) = \frac{Hb\ (g/dL) \times 10}{RBC\ (\times 10^{12}/L)}$$

$$MCHC = \frac{Hb\ (g/dL) \times 10}{PCV\ (\%)}$$

3.21 Determination of Total Plasma Protein

Total plasma protein was determined using the hand refractometric method (Sirois, 1995). After reading the PCV, each of the haematocrit capillary tubes was broken by snapping the tube at a point immediately above the buffy coat. The end containing thebuffycoat and packed red cells was discarded retaining the end containing the plasma. The plastic flap of the hand refractometer was reflected to exposed glassy sample surface which was thoroughly wiped with cotton wool. The broken end of the capillary tube was then used to touch the sample surface of the refractometer in order to place plasma sample unto the surface, after which, the capillary was discarded. The plastic flap of the refractometer was then replaced; pressure was then applied on the flap against the plasma sample with the index finger and holding the refractometer against light.The concentration of plasma proteins was then read in grams per decilitre (g/dL).

3.22 Determination of Serum Immunoglobulin Y and Cytokine Concentration

3.22.1 Determination of serum immunoglobulin Y concentration

Two millilitres of blood were collected from five birds each in all the groups through venipuncture using a needle and syringe into a serum separator tubes at day 14, 21, 28 and 35 of age representing the day ofprimary vaccination, booster dose, infection and euthanasia, respectively. The blood samples were allowed to clot for two hours at room temperature before centrifugation for 20 minutes at approximately 1000 x g to harvest the serum which was stored at -20 ^{o}C.

The prepared serum was transported in a cold box to theBiotechnology Laboratory of the Molecular Bacteriological Unit, National Veterinary Research Institute, Vom, to analyse for the level of serum immunoglobulin and cytokine. The concentration of serum immunoglobulin (IgY) was detected by using an indirect ELISA with the “Chicken Immunoglobulin Y (IgY) ELISA Kit” of Abbkine, Inc – China in duplicate according to manufacturer’s instructions.

3.22.2 Determination of serum interferon gamma concentration

The concentration of interferon gamma (IFN-γ) in serum of the chickens sampled in each group was detected by using an indirect ELISA with the “Chicken Interferon Gamma (IFN-γ) ELISA Kit" of Abbkine, Inc – China in duplicate according tomanufacturer's instructions.

3.23 Assessment of Caecal Gross Lesions and Weight

3.23.1 Determination of caecal lesion score

Lesion score was obtained seven days post-inoculation. Five birds from each group were euthanized and caecal lesion score was evaluated as described by Johnson and Reid (1970). Grades from 0–4 covers a range of gross lesions which were allocated based on severity. The scoring was assigned as follows:

Score 0 = Absence of any visible gross lesions,

Score 1 = A few scattered petechiation on the serosal wall; No thickening of the caecal wall or blood present; Caecal contents appear normal,

Score 2 = Lesions more numerous with noticeable blood in the caecal contents; caecal wall somewhat thickened,

Score 3 = Large amount of blood or caecal cores present , caecal walls greatly thickened, little if any faecal contents in the caeca and,

Score 4 = Caecal wall greatly distended with blood or large caseous cores; faecal debris lacking or included in cores.

3.23.2 Determination of caecal weight

The caecal weight of each of the five (5) euthanized birds per group was determined using a laboratory weighing balance.

3.24 Histopathological Examination of the Caeca

Histopathological studies of the caecal tissues were carried out in the histopathological laboratory of the Department of Veterinary Pathology, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria according to the method described by Mitchell *et al*. (2009) as follows: Tissue samples (caeca) from the euthanized birds were fixed in10% neutral buffered formalin for at least 48 h. The fixed tissues were dehydrated in graded concentrations of alcohol (70%, 80%, 90%, 95% and 100%) at intervals of 1 h. The tissues were cleared with xylene for about 2 hours, infiltrated with molten paraffin wax at 50 ^{o}C to 60 ^{o}C for 2 h, embedded in the molten paraffin wax and labeled appropriately. Sections 5µ thick were cut from the embedded tissues using a microtome knife attached to a microtome. The sectioned tissues were mounted on grease free clean glass slides. They were then dried at room temperature and stained with haematoxylin and Eosin (H & E) stain. The tissue sectioned on slides were studied using light microscope at x200 and x400 magnifications. Photomicrographs of the slides were taken using a digital camera, transferred to a computer and labeled appropriately.

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3.25 Data Analyses

All data were expressed as the mean and standard error of mean (± SEM) of 10 birds per group, and all data were statistically analyzed by two-way analysis of variance (ANOVA), complimented by posthoc analysis using the Tukey's HSD test. All statistical analyses were processed by theSPSS13.0 Data Editor software (SPSS Inc., Chicago, IL). The differences between groupswere considered to be significant if Pvalues were less than 0.05. Data collected were presented in tables, graphs and photographs.

Table 3.2: Experimental design to evaluate the efficacy and immunogenicity of EtAMA1 DNA vaccine in broiler chickens experimentally infected with *Eimeria tenella*oocysts

Group	Treatment	No. of birds	DNA vaccine administered	Age when vaccinated (days)	Age when blood was collected (days)	*E. tenella*oocysts challenged with	Age wheninoculated/weighed (days)	Age when birds were euthanized (days)	No. of birds euthanized	Sample (s) collected
G1	Unvaccinated unchallenged	20	None	-	14, 21, 28, 35	None	Nil/28, 35	35	5	Blood and caeca
G2	Unvaccinated challenged with Local isolate	20	None	-	14, 21, 28, 35	*E. tenella* Local isolate	28/28, 35	35	5	Blood and caeca
G3	Vaccinated challenged with Local isolate	20	EtAMA1	14, 21	14, 21, 28, 35	*E. tenella* Local isolate	28/28, 35	35	5	Blood and caeca
G4	Vaccinated challenged with Houghton strain	20	EtAMA1	14, 21	14, 21, 28, 35	*E. tenella* Houghton strain	28/28, 35	35	5	Blood and caeca
Total		80							20	

CHAPTER FOUR

4.0 RESULTS

4.1 Expression of EtAMA1 Gene in Recombinant Plasmid DNA

Results of reverse transcription-polymerase chain reaction (RT-PCR) expressed the targeted gene, EtAMA1 from the recombinant plasmid DNA at a fragment of approximately 700-800 bp (Plate I).

4.2 Clinical Signs Observed

Signs of coccidiosis observed in chickens inoculated with *Eimeria* oocysts include depression, dullness, somnolence, reduced feed in-take, projectile bloody-watery diarrhoea, soiled vent, and morbidity (Plate II). Clinical signs initially appeared at3 dpi in group G2 (21-40%) and then in G3 (01-20%) and G4 (01-20%) on the 4 dpi. All the observable clinical signs were seen to peak on the 5 dpi for G2 (61-80%), G3 (21-40%) andG4 (01-20%). The clinical signs were very severe in G2 (61-80%), moderate in G3 (21-40%), and mild in G4 (01-20%). There was no clinical sign observed in G1 birds. (Table 4.1).

4.3 Body Weight Gain

At day 35 of age (7 days post infection) there was no significant ($P > 0.05$) difference in the average weight gain between birds in G3 (560 ± 10.55 g) and G4 (565 ± 14.70 g) but with a significant ($P < 0.05$) difference compared to G1 (640.00 ± 9.86 g) and G2 (490 ± 3.55 g) birds (Table 4.2 and Figure 4.1).

4.4 Feed Conversion Ratio

The mean feed conversion ratios showed no statistical significant ($P > 0.05$) difference between birds in G1 (3.48 ± 0.18), G3 (3.47 ± 0.25) and G4 (3.46 ± 0.18) but significantly ($P < 0.05$) lower than the G2 (3.73 ± 0.19) chickens (Table 4.2).

4.5 Survival Rate

The survival rate was 100% for all chickens in all the groups (Table 4.3).

4.6 Oocyst Decrease Ratio

The oocyst decrease ratios in G1 (unvaccinated unchallenged), G2 (unvaccinated challenged), G3 (vaccinated + Local isolate) and G4 (vaccinated + Houghton strain) were 100 %, 0.00 %, 81.33 % and 75.00 %, respectively (Table 4.3).

4.7 Anticoccidial Index

The anticoccidial indices values were as shown: G1 (unvaccinated unchallenged), G2 (unvaccinated challenged), G3 (vaccinated + Local isolate) and G4 (vaccinated + Houghton strain) 200, 72.56, 165.83 and 160.28, respectively (Table 4.3).

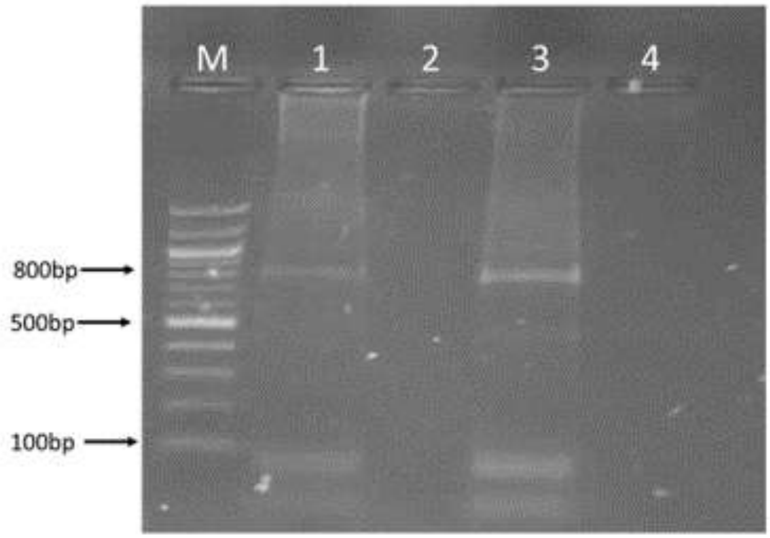

Plate I: Detection of expression of EtAMA1 DNA fragment by RT-PCR. LaneM–DL1000DNAmarker. Lane 1 – positive control. Lanes 2 – blank. Lane3– recombinant DNA showingtranscriptionofEtAMA1.Lanes 4 – negative control.

Plate II: Photograph of unvaccinated challenged group (G2) showing bloody faeces (arrow).

Table 4.1: Clinical signs in percentage, on a scale of 1 to 5, and grade of broiler chickens immunized with *Eimeria tenella* apical membrane antigen-1(EtAMA1) DNA vaccine and challenged with local isolate or Houghton strain of *Eimeria tenella*

Group	Treatment	Percentage (%)	Scale	Grade
G1	Unvaccinated unchallenged	Nil	Nil	Nil
G2	Unvaccinated challenged with local isolate	61 - 80	4	Very severe
G3	Unvaccinated challenged with local isolate	21 - 40	2	Moderate
G4	Unvaccinated challenged with Houghton strain	01 - 20	1	Mild

Table 4.2: Mean body weight gain and feed conversion ratio of broiler chickens immunized with *Eimeria tenella* apical membrane antigen-1(EtAMA1) DNA vaccine and challenged with local isolate or Houghton strain of *Eimeria tenella*

	Group			
	G1	G2	G3	G4
	Treatment			
Parameters	Unvaccinated unchallenged	Unvaccinated challenged	Vaccinated + local isolate	Vaccinated + Houghton strain
Initial weight (g)	1,590.00 ± 34.80	1,520.00 ± 18.56	1,530.00 ± 32.66	1,560.00 ± 33.17
Final weight (g)	2,230.00 ± 24.94	2,010.00 ± 22.11	2,090.00 ± 22.11	2,125.00 ± 47.87
Weight gain (g)	640.00 ± 9.86^{a}	490 ± 3.55^{b}	560 ± 10.55^{c}	565 ± 14.70^{c}
Feed consumed (g)	2,228.57 ± 80.81	1,828.57 ± 80.81	1,942.86 ± 104.30	1,957.14 ± 57.14
Feed conversion ratio (FCR)	3.48 ± 0.18^{a}	3.73 ± 0.19^{b}	3.47 ± 0.25^{a}	3.46 ± 0.18^{a}

Values with the different superscript alphabets along the same row are statistically significant ($P < 0.05$).

Table 4.3: Survival rates and oocyst decrease ratios of broiler chickens immunized with *Eimeria tenella* apical membrane antigen-1(EtAMA1) DNA vaccine and challenged with Local isolate or Houghton strain of *Eimeria tenella*

Group	Treatment	Survival rate (%)	Oocyst output (x 10^5)	Oocyst decrease ratio (%)	Anticoccidial index (ACI)
G1	Unvaccinated unchallenged	100	0.00	100.00	200.00
G2	Unvaccinated challenged	100	19.76	0.00	72.56
G3	Vaccinated + Local isolate	100	3.69	81.33	165.83
G4	Vaccinated + Houghton strain	100	4.94	75.00	160.28

4.8 Changes in Haematological Parameters

4.8.1 Packed cell volume

There was no significant difference ($P > 0.05$) in the packed cell volumes (PCV) in all the chickens pre-challenge (Figure 4.2). The PCV significantly ($P < 0.05$) decreased in G2 (19.8 ± 0.66%) compared toG1 (29.00 ± 0.94%), G3 (28.40 ± 1.08%) and G4 (28.80 ± 0.84%) chickens 7 days post challenge (Figure 4.2).

4.8.2 Haemoglobin concentration

Pre-challenge, no significant difference ($P > 0.05$) in haemoglobin concentration was observed in all chickens (Figure 4.3). Seven days post-challenge, the haemoglobin concentrations showed no significant differences in G1 (9.68 ± 0.27 g/dl), G3 (9.42 ± 0.21 g/dl) and G4 (9.58 ± 0.45 g/dl) chickens but significantly ($P < 0.05$) became lower in G2 chickens (6.60 ± 0.22 g/dl) (Figure 4.3).

4.8.3 Total red blood cell count

The total red blood cell (TRBC) count in the various group of birds showed no statistical significant difference ($P > 0.05$) on day 0 post-challenge (Figure 4.4). Seven days post-challenge, the TRBC was significantly ($P < 0.05$) reduced in the G2 chickens (3.32 ± 0.13 x 10^6/µl) compared to the G1 (4.62 ± 0.14 x 10^6/µl), G3 (4.50 ± 0.11 x 10^6/µl) and G4 (4.48 ± 0.16 x 10^6/µl) chickens (Figure 4.4).

4.8.4 Mean corpuscular volume

There was no statistical significant difference ($P > 0.05$) in the mean corpuscular volume (MCV) among the groups of chickens atday 0 post-challenge (Figure 4.5). In G2 chickens, the MCV

(59.70 ± 0.51*fl*) significantly ($P < 0.05$) decreased at day 7 post-challenge compared to G1 (62.94 ± 2.31 *fl*), G3 (63.19 ± 2.37*fl*) and G4 (63.82 ± 3.05*fl*) chickens (Figure 4.5).

4.8.5 Mean corpuscular haemoglobin

At day 0 post-challenge, there was no statistical significant difference ($P > 0.05$) in mean corpuscular haemoglobin (MCH) between the various groups of chickens (Figure 4.6). Seven dayspost-challenge, there was a significant decrease in G2 (19.90 ± 0.21) chickens compared toG1 (21.01 ± 0.74), G3 (21.06 ± 0.79) and G4 (21.45 ± 1.08) chickens (Figure 4.6).

4.8.6 Total white blood cell count

There was no statistical significant difference ($P > 0.05$) in the total white blood cells (TWBC) between the group of birds at day 0 post-challenge (Figure 4.7). The TWBC significantly ($P < 0.05$) increased 7 days post infection inG2 chickens (22.22 ± 0.68 x 10^3/µl) compared to the G1 (20.88 ± 0.21 x 10^3/µl), G3 (21.00 ± 0.47 x 10^3/µl) and G4 (21.58 ± 0.49 x 10^3/µl) chickens (Figure 4.7).

4.8.7 Heterophil count

The heterophil count showed no statistical significant difference ($P > 0.05$) among the groups of chickens at day 0 post-challenge (Figure 4.8). In G2 chickens the heterophil count (8.92 ± 0.60 x 10^3/µl) was significantly ($P < 0.05$) higher 7 days post-challenge compared to G1 (7.90 ± 0.36 x 10^3/µl), G3 (7.61 ± 0.34 x 10^3/µl) and G4 (8.02 ± 0.27 x 10^3/µl) chickens (Figure 4.8).

4.8.8 Lymphocyte count

The lymphocyte count showed no statistical significant difference ($P > 0.05$) between the groups ofchickens day 0 post-challenge (Figure 4.9). In G1 (12.81 ± 0.30 x 10^3/µl), G3 (13.14 ± 0.31 x

$10^3/\mu l$) and G4 ($13.44 \pm 0.57 \times 10^3/\mu l$) chickens the lymphocytes count was not significantly ($P > 0.05$) increase compared to G2 ($12.44 \pm 0.53 \times 10^3/\mu l$) (Figure 4.9).

4.8.9 Heterophil/lymphocyte ratio

At day 0 post-challenge, there was no statistical significant difference ($P > 0.05$) in heterophil/lymphocyte ratio among the various groups of birds (Figure 4.10). Also, there was no statistical significant difference in heterophil/lymphocyte ratio observed in G1 (0.62 ± 0.04), G3 (0.58 ± 0.03) and G4 (0.60 ± 0.04) 7 days post-challenge. In G2 chickens, the heterophil/lymphocyte ratio (0.72 ± 0.06) significantly ($P < 0.05$) increased 7 days post-challenge (Figure 4.10).

4.9 Changes in Total Plasma Protein Concentration

All chickens showed no significant difference in total plasma protein concentration pre-challenge (Figure 4.11). In G2 chickens, the total protein (3.44 ± 0.15 g/dl) significantly ($P < 0.05$) decreased 7 days post-challenge compared to G1 (4.54 ± 0.09 g/dl), G3 (4.42 ± 0.11 g/dl) and G4 (4.30 ± 0.15 g/dl) chickens (Figure 4.11).

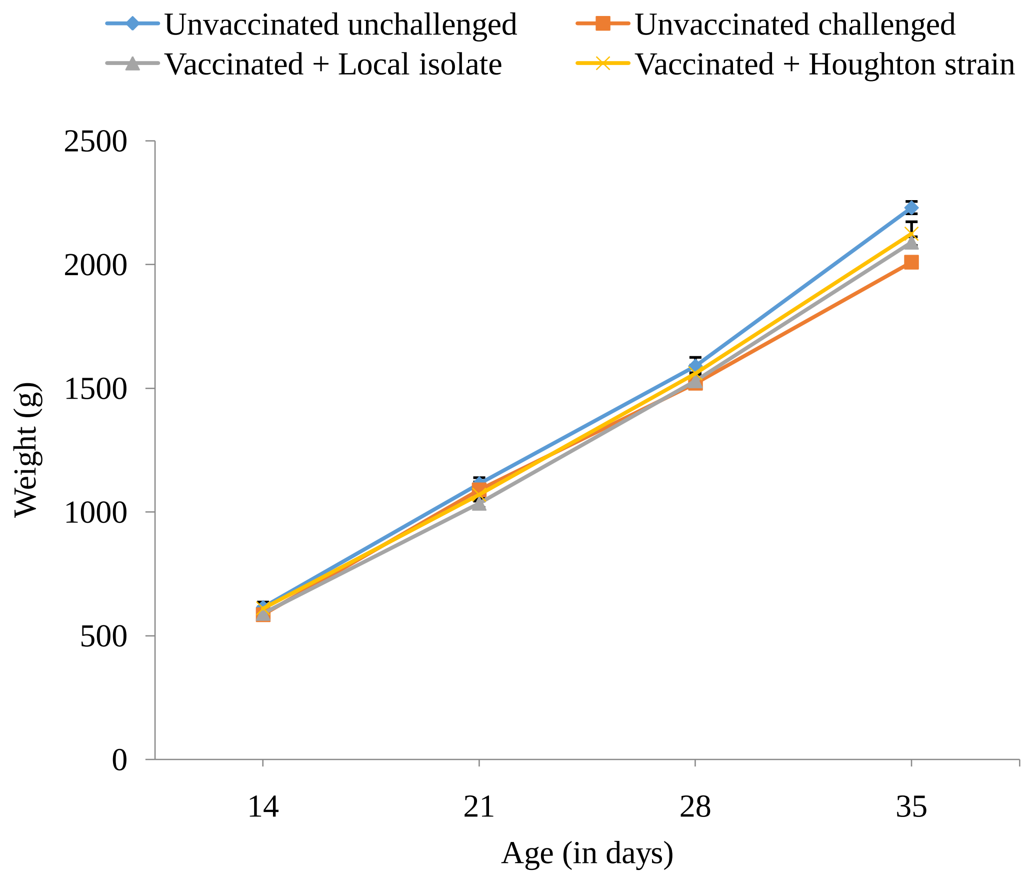

Figure 4.1: Mean body weights of broiler chickens immunized with *Eimeria tenella* apical membrane antigen-1(EtAMA1) DNA vaccine and exposed to local isolate or Houghton strain of *Eimeria tenella*.

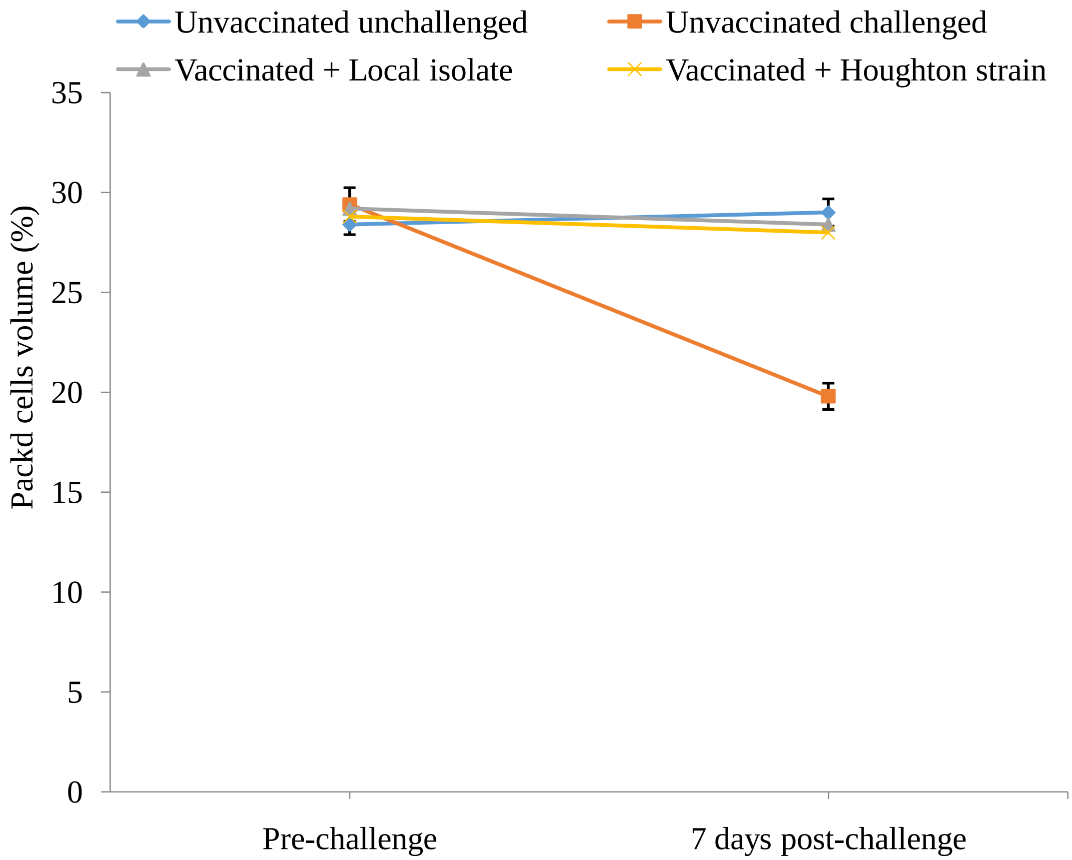

Figure 4.2: Packed cell volume of broiler chickens immunized with *Eimeria tenella* apical membrane antigen-1(EtAMA1) DNA vaccine and exposed to local isolate or Houghton strain of *Eimeria tenella*.

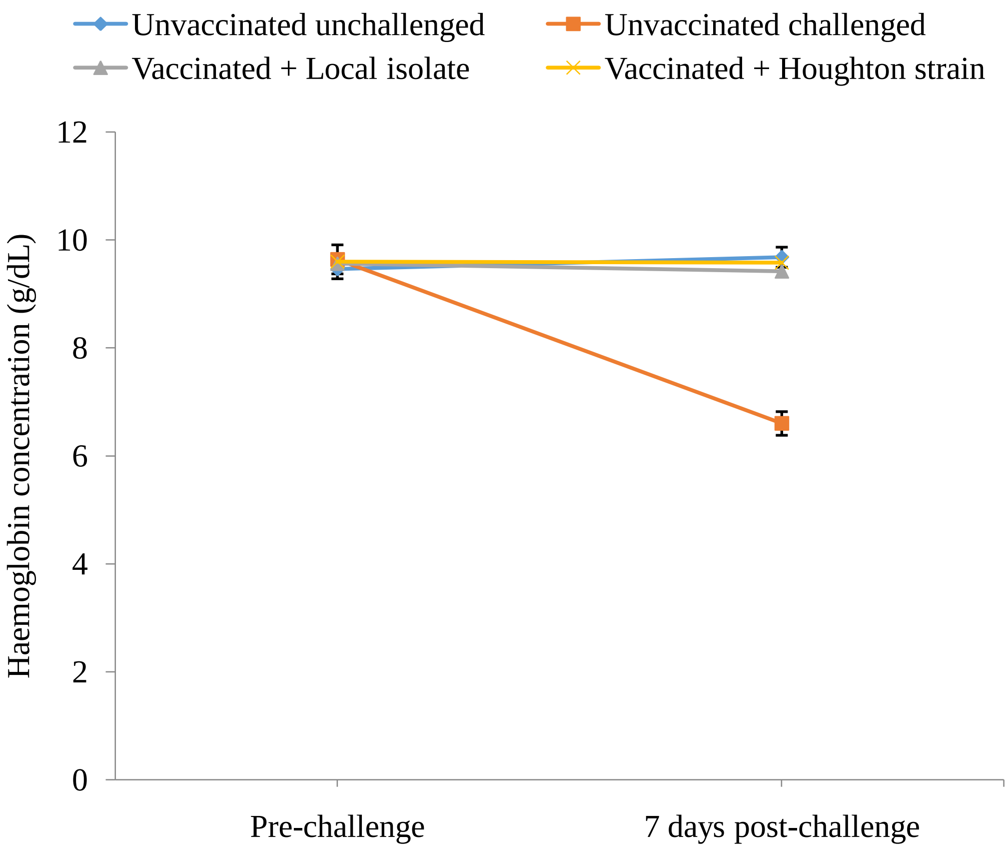

Figure 4.3: Haemoglobin concentration of broiler chickens immunized with *Eimeria tenella* apical membrane antigen-1(EtAMA1) DNA vaccine and exposed to local isolate or Houghton strain of *Eimeria tenella*.

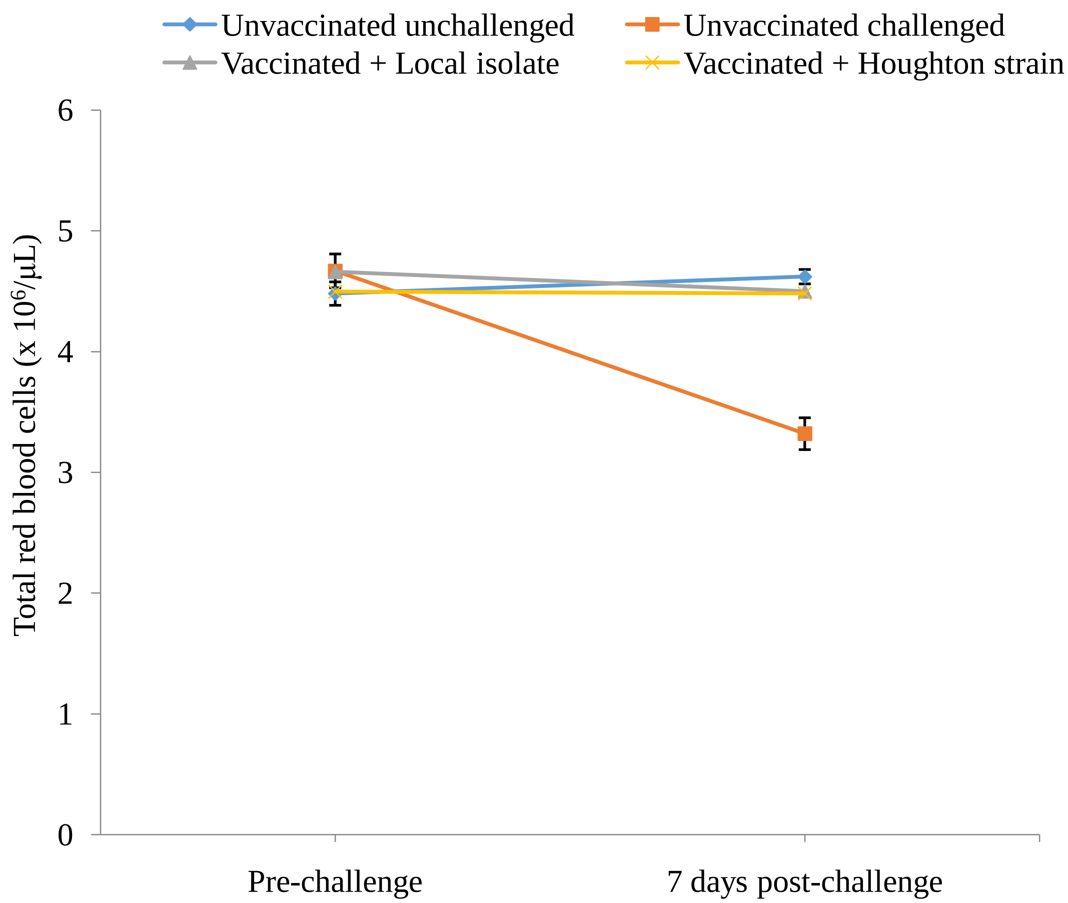

Figure 4.4: Total red blood cell count of broiler chickens immunized with *Eimeria tenella* apical membrane antigen-1(EtAMA1) DNA vaccine and exposed to local isolate or Houghton strain of *Eimeria tenella*.

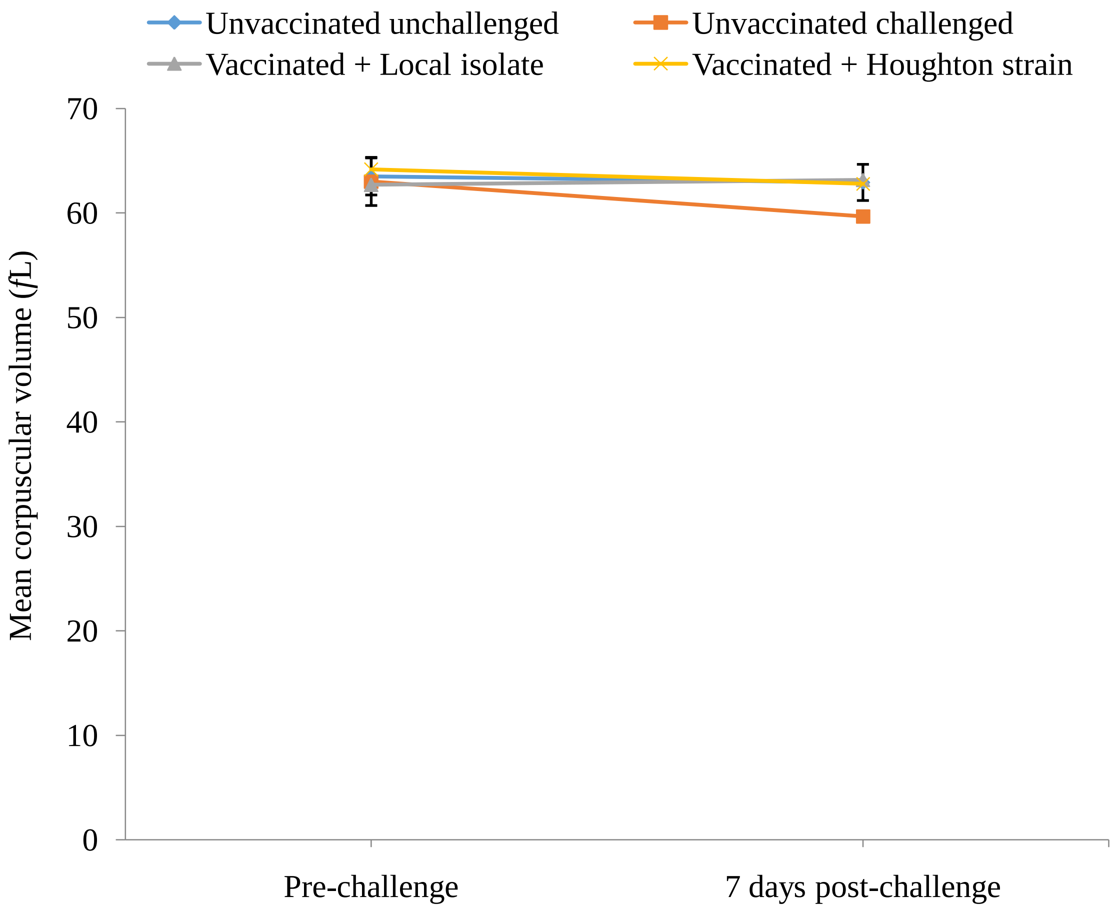

Figure 4.5: Mean corpuscular volume of broiler chickens immunized with *Eimeria tenella* apical membrane antigen-1(EtAMA1) DNA vaccine and exposed to local isolate or Houghton strain of *Eimeria tenella*.

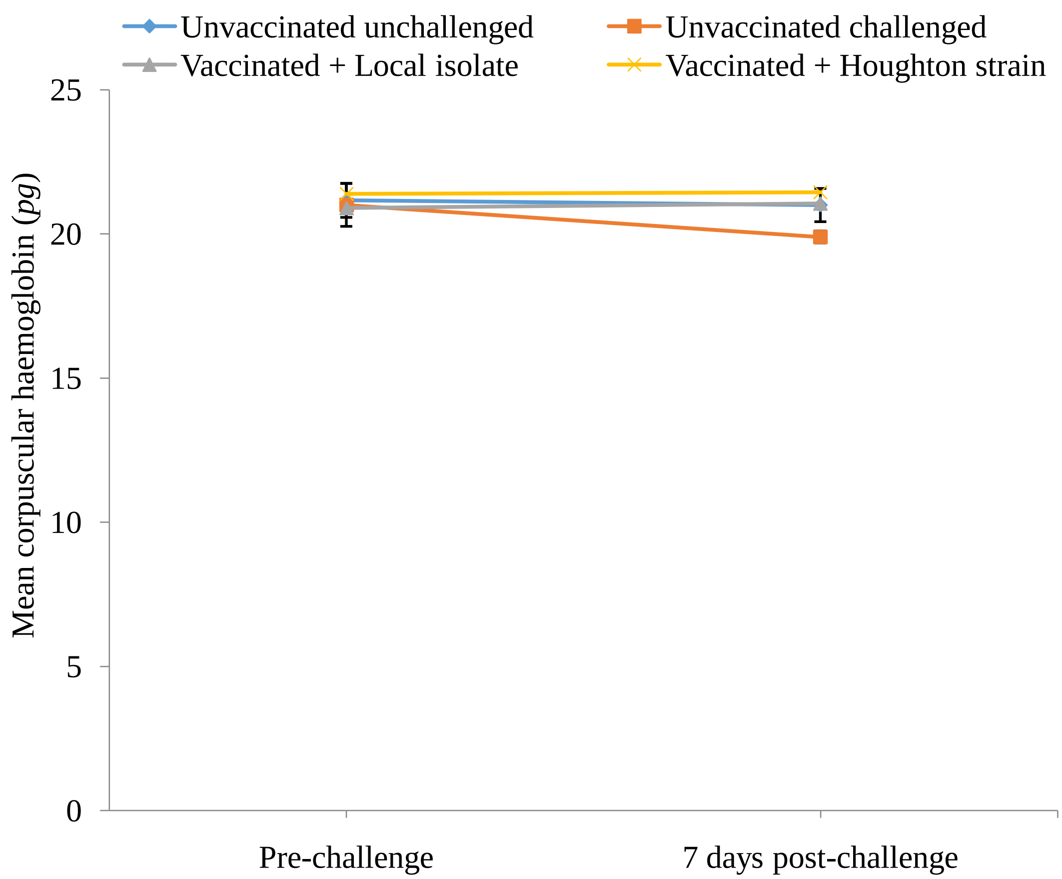

Figure 4.6: Mean corpuscular haemoglobin of broiler chickens immunized with *Eimeria tenella* apical membrane antigen-1(EtAMA1) DNA vaccine and exposed to local isolate or Houghton strain of *Eimeria tenella*.

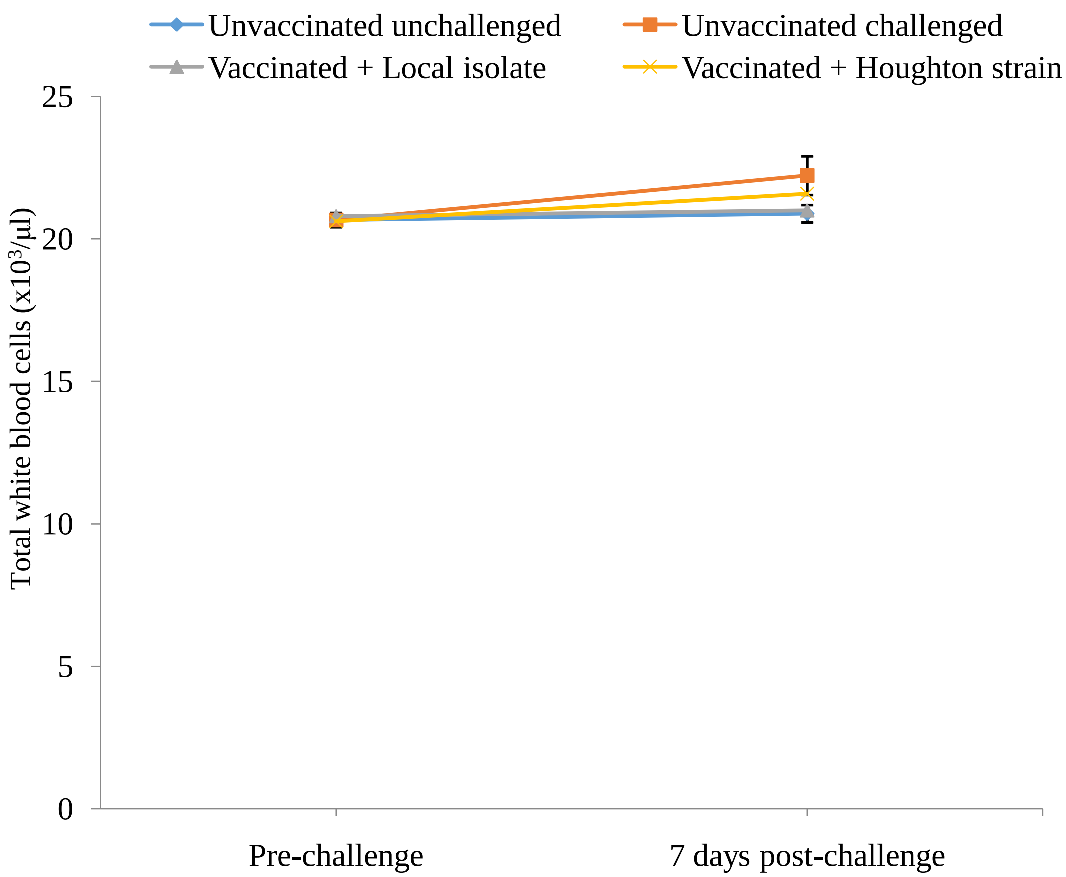

Figure 4.7: Total white blood cell count of broiler chickens immunized with *Eimeria tenella* apical membrane antigen-1(EtAMA1) DNA vaccine and exposed to local isolate or Houghton strain of *Eimeria tenella*.

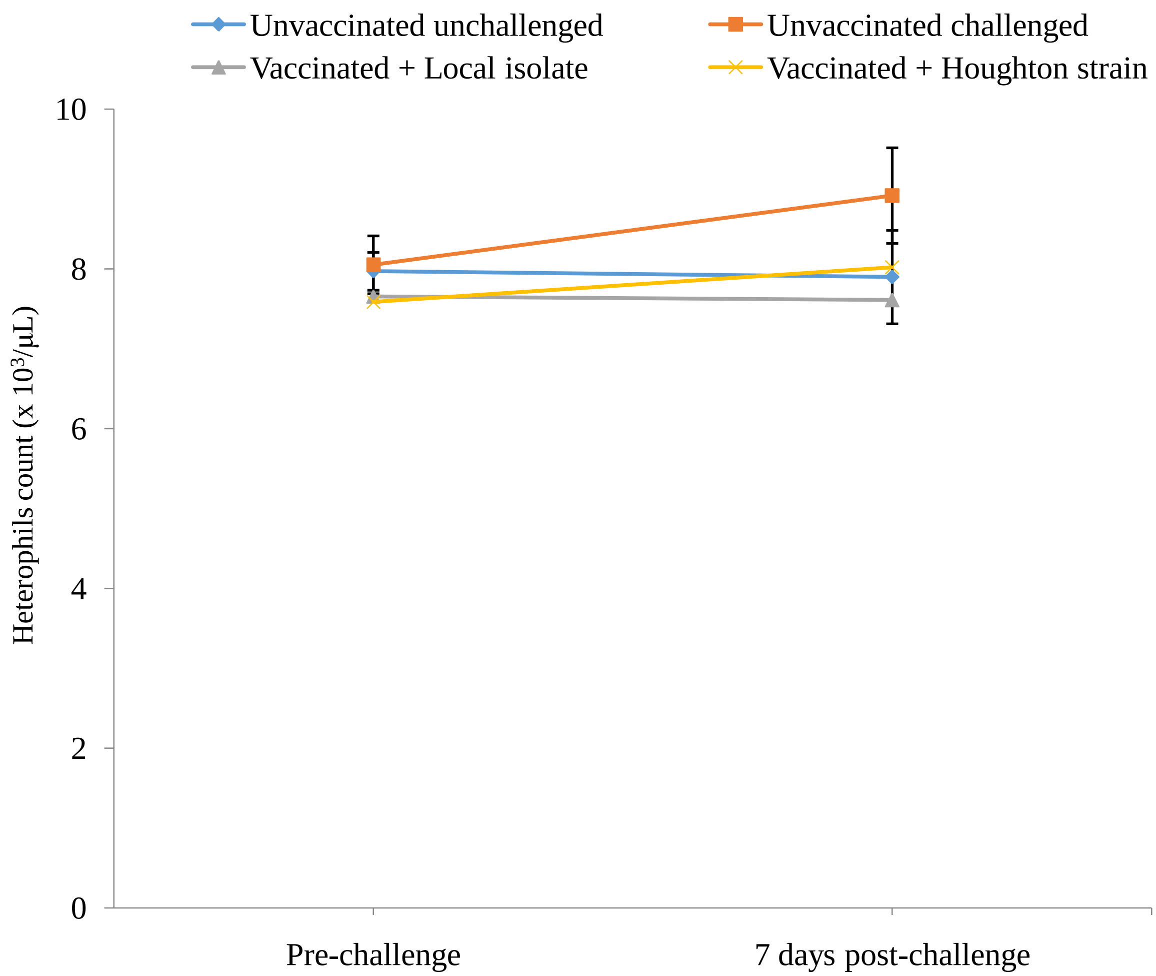

Figure 4.8: Heterophil count of broiler chickens immunized with *Eimeria tenella* apical membrane antigen-1(EtAMA1) DNA vaccine and exposed to local isolate or Houghton strain of *Eimeria tenella*.

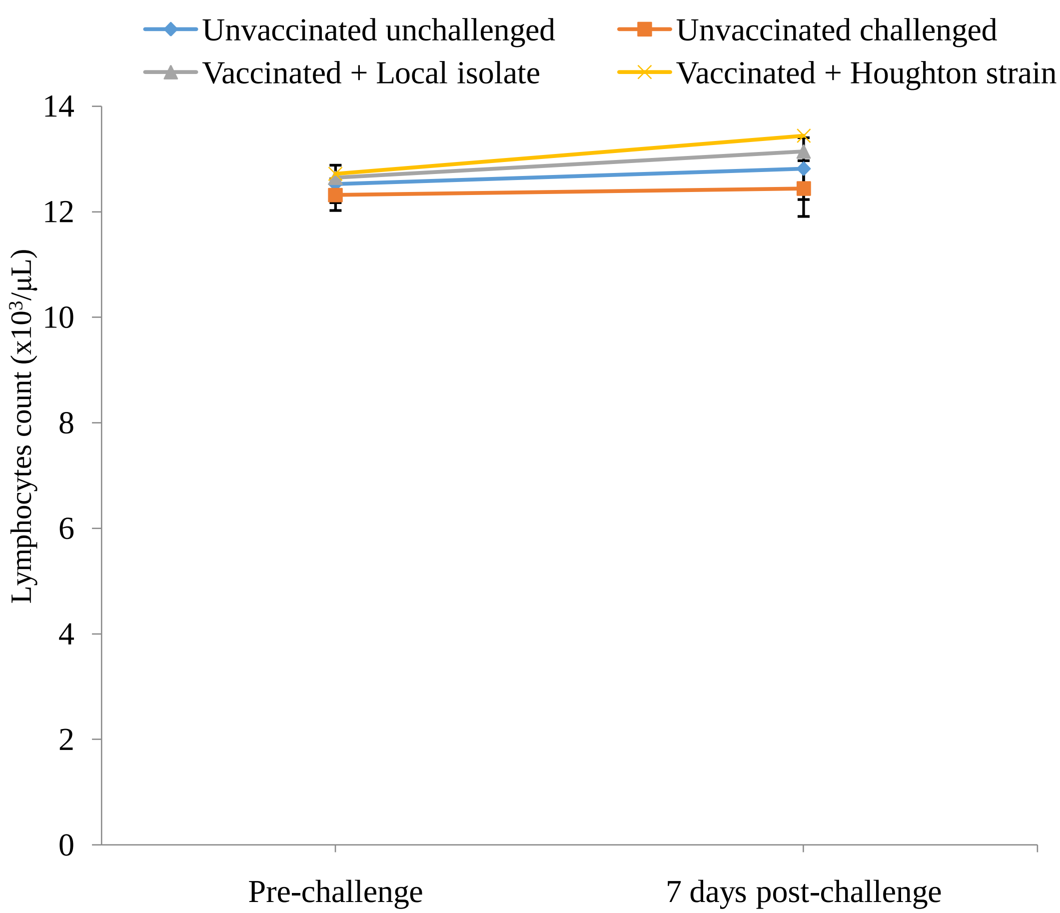

Figure 4.9: Lymphocyte count of broiler chickens immunized with *Eimeria tenella* apical membrane antigen-1(EtAMA1) DNA vaccine and exposed to local isolate or Houghton strain of *Eimeria tenella*.

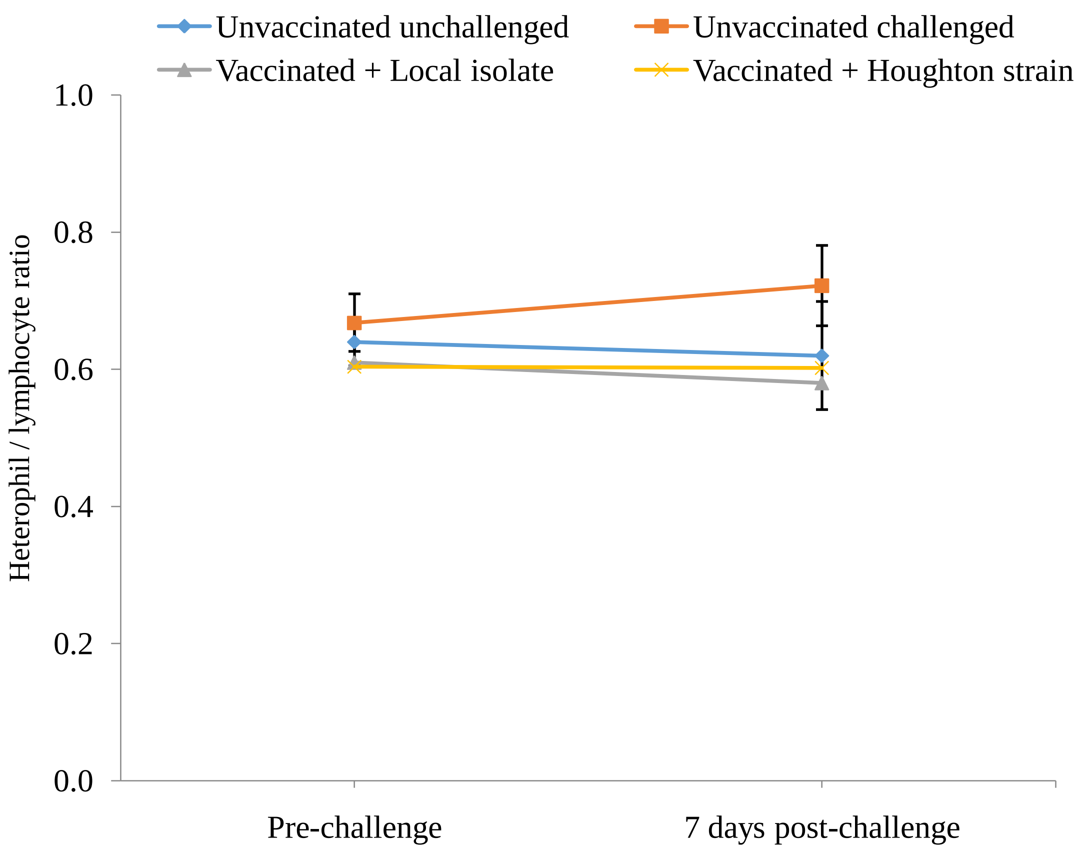

Figure 4.10: Heterophil/lymphocyte ratio of broiler chickens immunized with *Eimeria tenella* apical membrane antigen-1(EtAMA1) DNA vaccine and exposed to local isolate or Houghton strain of *Eimeria tenella*.

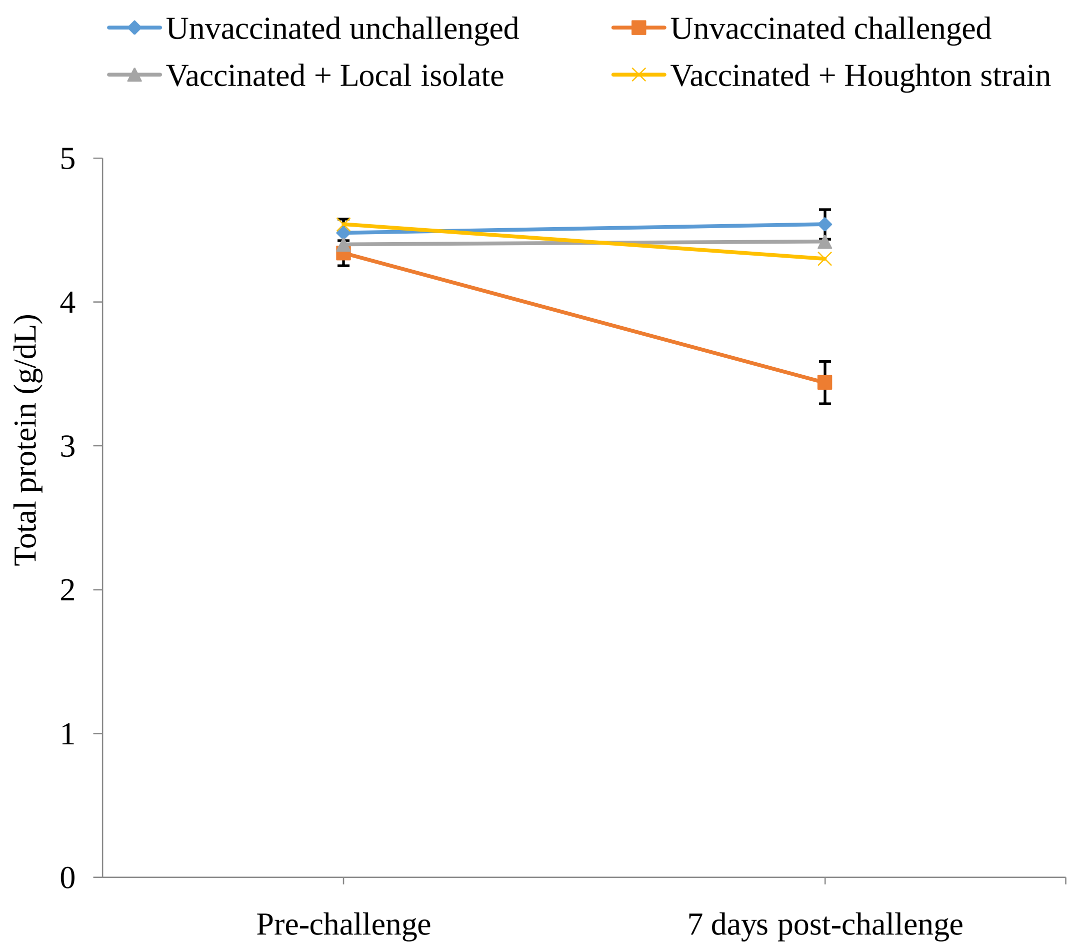

Figure 4.11: Total protein concentration of broiler chickens immunized with *Eimeria tenella* apical membrane antigen-1(EtAMA1) DNA vaccine and exposed to local isolate or Houghton strain of *Eimeria tenella*.

4.10 Serum Immunoglobulin and Cytokine Concentrations

4.10.1 Changes in serum level of immunoglobulin-Y

There was no significant difference (P > 0.05) in the serum level of immunoglobulin Y (IgY) in all the chickens prior to primary vaccination on day 14 of age (Figure 4.12). However, after a booster vaccination on day 21, the serum level of IgY significantly (P < 0.05) increased in G3 (6.08 ± 0.16 μg/ml) and G4 (5.96 ± 0.09 μg/ml) chickens reaching a peak of 11.70 ± 0.23 μg/ml and 11.64 ± 0.23 μg/ml, respectively at day 28 of age. Following challenge, a significant decrease (P > 0.05) was observed in G2 (1.05 ± 0.02 μg/ml), G3 (9.16 ± 0.16 μg/ml) and G4 (7.78 ± 0.18 μg/ml) chickens at day 35 (Figure 4.12).

4.10.2 Changes in serum level of interferon-ϒ

The serum level of interferon-ϒ (IFN-ϒ) showed no significant (P > 0.05) difference in the chickens at day 14 of age before vaccination (Figure 4.13). At days 21 and 28, the serum level of IFN-ϒ significantly (P < 0.05) increased in G3 (40.83 ± 0.50 pg/ml; 62.60 ± 0.57 pg/ml) and G4 (38.85 ± 0.48 pg/ml; 61.62 ± 0.50 pg/ml) chickens compared to G1 (1.41 ± 0.21 pg/ml; 1.50 ± 0.18 pg/ml) and G2 (1.34 ± 0.19pg/ml; 1.34 ± 0.02 pg/ml) chickens. Seven (7) days post vaccination booster further significant (P < 0.05) increase in serum level of IFN- ϒ was observed at day 35 in G3 (116.30 ± 0.28 pg/ml) and G4 (115.90 ± 0.44 pg/ml) chickens compared to chickens in G2 (0.99 ± 0.04 pg/ml) (Figure 4.13).

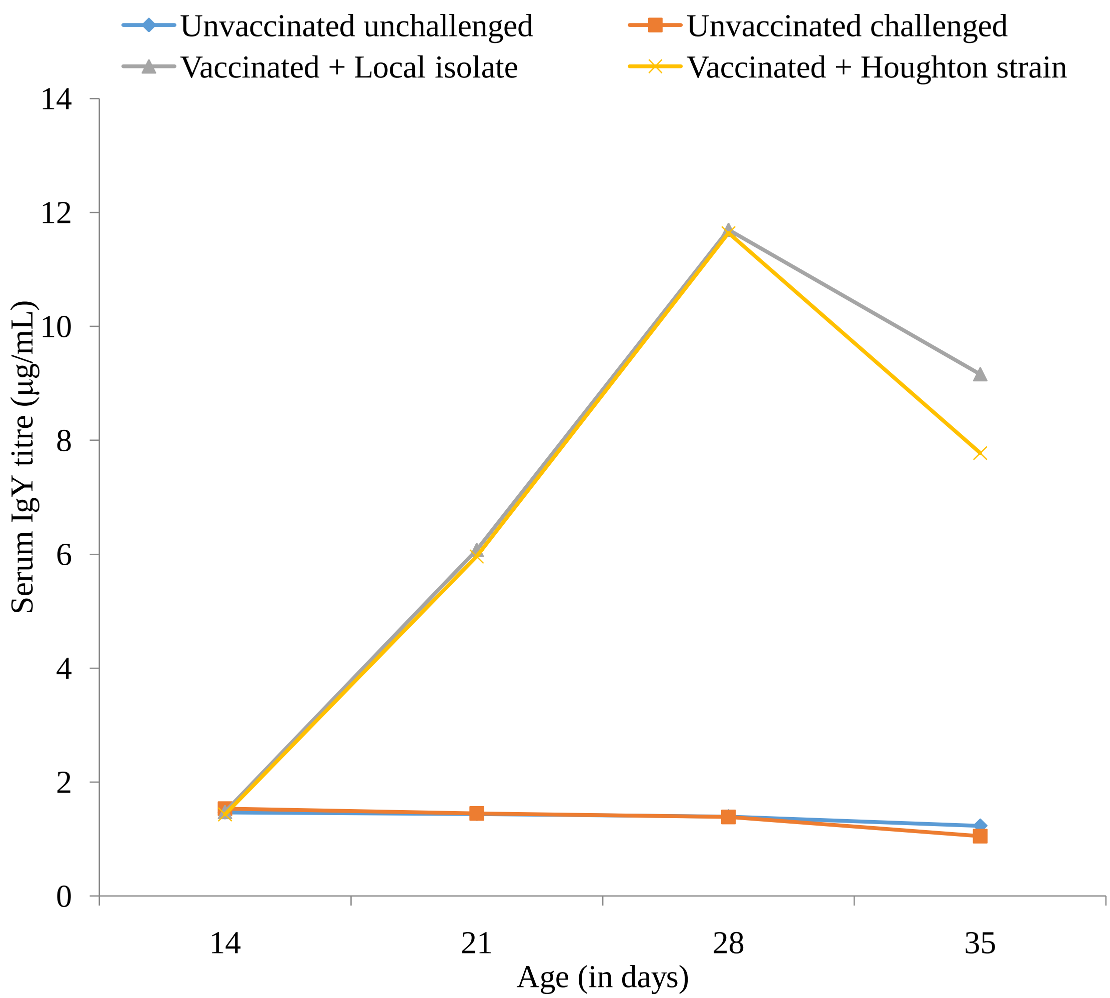

Figure 4.12: Serum immunoglobulin-Y levels of broiler chickens immunized with *Eimeria tenella* apical membrane antigen-1(EtAMA1) DNA vaccine and challenged withLocal isolate or Houghton strain of *Eimeria tenella*.

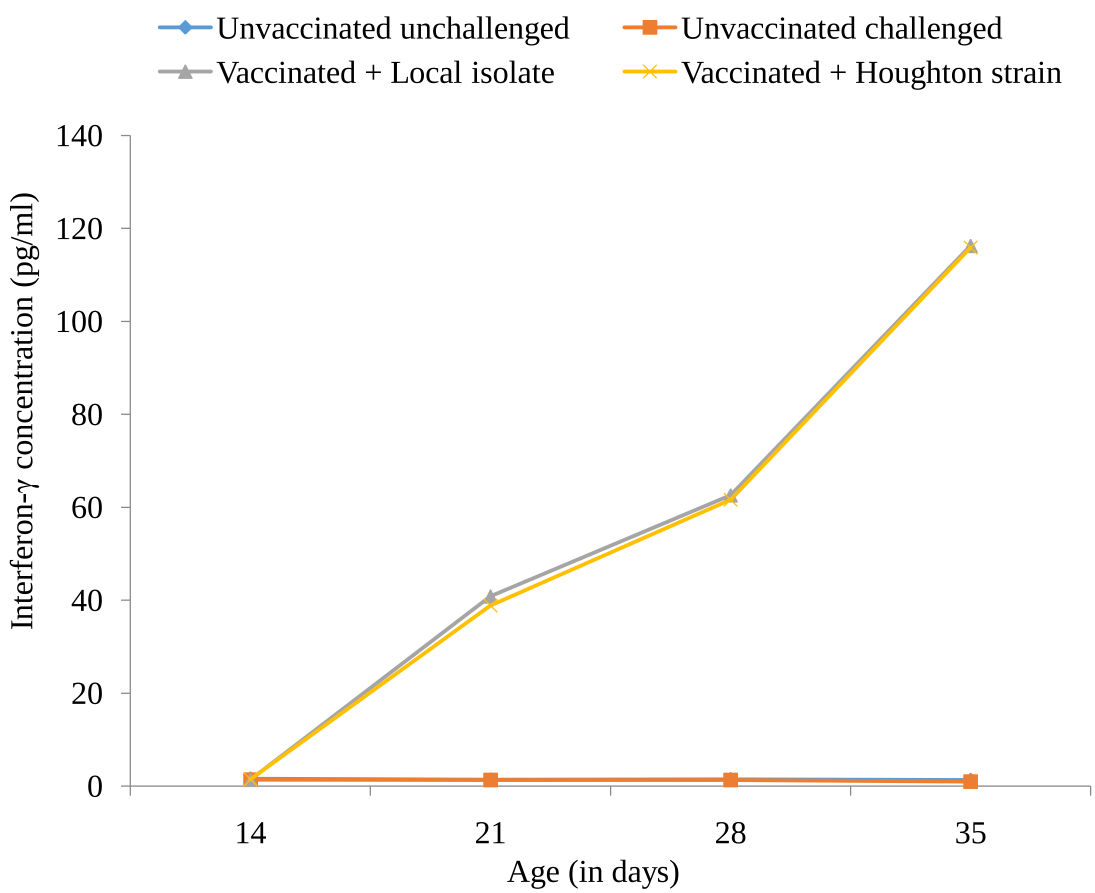

Figure 4.13: Serum interferon-ϒ levels of broiler chickens immunized with *Eimeria tenella* apical membrane antigen-1(EtAMA1) DNA vaccine and challenged with Local isolate or Houghton strain of *Eimeria tenella*.

4.11 Gross and Microscopic Lesions in the Caeca

4.11.1 Caecal weight

The mean caecal weight of G1 chickens (4.61 ± 0.02 g) was significantly ($P < 0.05$) higher compared to G3 (3.95 ± 0.07), G4 (4.06 ± 0.03) and G2 (3.25 ± 0.09) chickens (Figure 4.14).

4.11.2 Caecal gross lesions

Caecal gross lesions were absent in G1 (score of 0), moderate in G3 and G4 (score of 2) and very severe in G2 (score of 4) (Table 4.4). The caecal gross lesions observed were petechial haemorrhages on serosal wall, thickened caecal wall, bloody caecal content, shortened caecal length, narrow caecal width, caecal core and sloughing of caecal mucosae (Table 4.4).

4.11.3 Caecal microscopic lesions

The caeca of G1 (unvaccinated unchallenged) chickens showed intact mucosal glands and tissue architecture (Plate III). In G2 (unvaccinated challenged) chickens, there was desquamation of the epithelium and necrotic mucosal glands in the caecum (Plate IV). In chickens immunized with *Eimeria tenella* apical membrane antigen-1(EtAMA1) DNA vaccine and exposed to local isolate (G3) or Houghton strain of *Eimeria tenella*(G4), the caeca showed intact mucosal glands and tissue architecture (Plates V and VI).

Table 4.4: Gross lesion score of broiler chickens immunized with *Eimeria tenella* apical membrane antigen-1(EtAMA1) DNA vaccine and challenged with local isolate or Houghton strain of *Eimeria tenella*

Group	Lesion	Score	Grade
G1	No lesions	0	Absent
G2	Numerous petechiae on serosal wall, thickened caecal wall, bloody caecal content, sloughing of caecal mucosae and caecal cores	+4	Very severe
G3	Few petechiae on serosal wall, thickened caecal wall, bloody caecal content	+2	Moderate
G4	Few petechiae on serosal wall, slight bloody caecal content	+2	Moderate

Key: 0=Absent;+1=Mild;+2=Moderate;+3=Severe; +4=Very severe

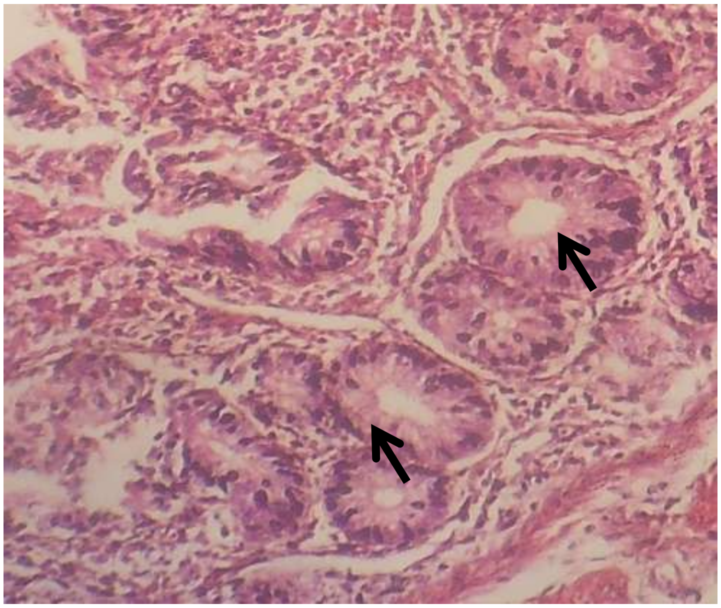

Plate III: Photomicrograph of the section of a caecum from unvaccinated unchallenged broiler chicken showing intact mucosal glands (arrows) and tissue architecture. H & E x 200.

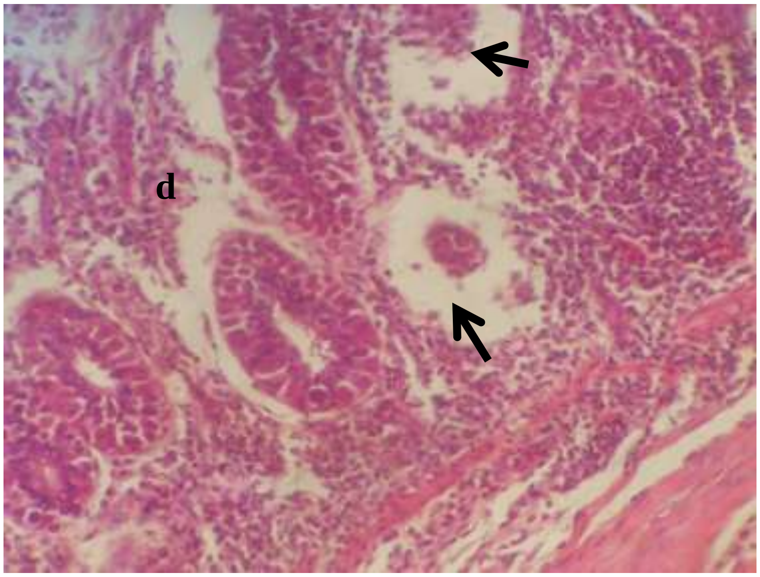

Plate IV: Photomicrograph of the section of a caecum from unvaccinated challenged broiler chicken showing necrotic mucosal glands (arrows) and desquamation of the epithelium (d). H & E x 200.

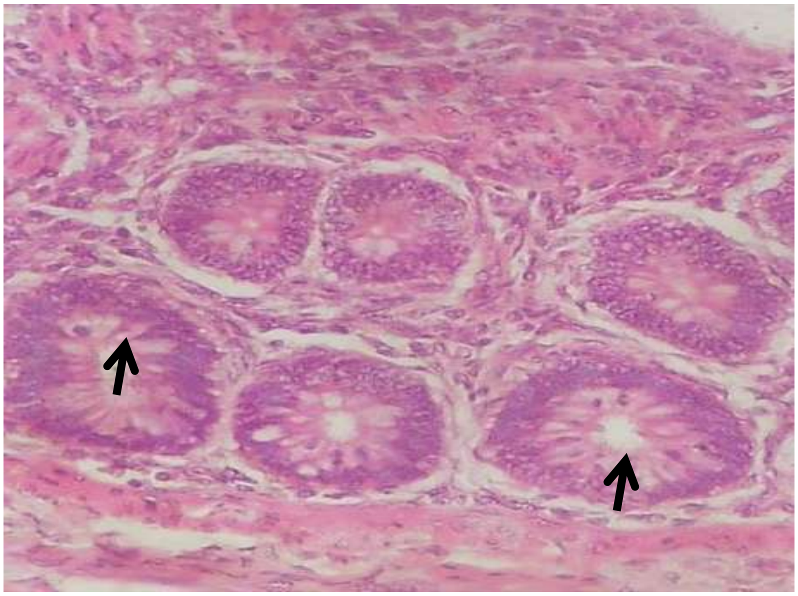

Plate V: Photomicrograph of the section of a caecum of a broiler chicken immunized with *Eimeria tenella* apical membrane antigen-1(EtAMA1) DNA vaccine and challenged with Local isolate of *Eimeria tenella*. Note intact mucosal glands (arrows). H & E x 200.

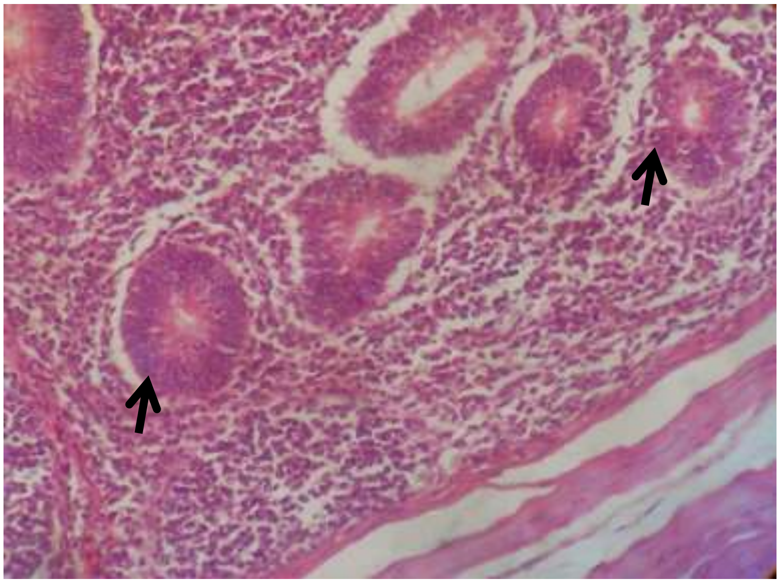

Plate VI: Photomicrograph of the section of a caecum of broiler chicken immunized with *Eimeria tenella* apical membrane antigen-1(EtAMA1) DNA vaccine and challenged with Houghton strain of *Eimeria tenella*. Note intact mucosal glands (arrows). H & E x 200.

CHAPTER FIVE

5.0 DISCUSSION

AMA1 antigen has been considered for use to confer universal protection against field strains of *Eimeria* species when incorporated in vaccine development (Blake *et al.,* 2015). The present study was conducted to evaluate the efficacy and immunogenicity of the homologous EtAMA1 DNA vaccine against broiler chickens experimentallyinfected with a heterologous*E. tenella* local isolate. Clinical signs associated with coccidiosis were very severe in G2, moderate in G3 and mild in G4 according to Kilyobas (2018) grading. Rafiqi *et al.* (2018) reported similar findings in broiler chickens vaccinated against *E. tenella* using *E.tenella* recombinant refractile body protein, rEtSO7. Prior to challenge, the mean body weights of the chickens in all the groups showed no significant($P > 0.05$) difference. However, 7 days post infection the average weight gain in G2 were significantly ($P < 0.05$) less compared to G1, G3 and G4. Similar increase in body weight gain has been reported by Zhu *et al.* (2012) and Song *et al.* (2013). Another homologous challenge experiment was performed by Hoan *et al*. (2014) in chickens after recombinant EtIMP1-C vaccination in which the average weight gain in vaccinated birds was higher compared to unvaccinated challenged birds. The feed conversion ratios showed no significant ($P > 0.05$) difference between birds in G1, G3 and G4 but significantly ($P < 0.05$) lower than in G2 chickens. The survival rate was 100% for all the groups of birds in the present study, no chicken died. There may be two possibilities for this. One may be that the mortality is not the detriment of the *E. tenella* coccidiosis. The other may be that challenge could be too weak to cause any kind of mortality. However, an acceptable standard of challenge infection experiment is a significant reduction (25%) in the rate of weight gain and a significant increase (2.5 units) in lesion scores over the non-infected controls. The body weight gain of the group of challenged control was depressed by about 23% compared with the unchallenged control and the mean lesion score of the group of challenged control was 3, which indicated that the challenge dose was quite appropriate.Similar kind of observation was made by Song *et al*. (2009) that no chicken died following homologous coccidiosis challenge infection. The oocyst decrease ratios in G1, G2, G3, and G4 was 100%, 0.00%, 81.33% and 75.00%, respectively. The oocyst output upon challenge had considerably reduced in the vaccinated birds. As the reduction in oocyst output was significantly high (81.33%), it will help in reducing the disease burden on farm by lowering the number of birds exposed to the pathogen. Moreover, the few number of re-circulating oocysts will further help in establishing immunity in the birds (Sathish *et al*., 2011). The anticoccidial index (ACI) value of chickens in G3and G4 respectively was more than 160, suggesting a good protection. However, the ACIs of unvaccinated challenged chickens, G2 was less than 160 indicating no protection. (McManus *et al*., 1968).

The PCV, Hb concentration, total red blood cell (TRBC) count, and total plasma protein was significantly ($P < 0.05$) less in G2 compared to the G1, G3 and G4 chickens 7 days post challenge. The decrease in PCV, Hb concentration, total red blood cell and total plasma protein which led to normocytic normochromic anaemia could be due to haemorrhages caused by the *Eimeria tenella* infection. This result is similar to Adamu *et al. (*2013), who reported that coccidiosis causes anaemia characterized by decrease in PCV, Hb concentration, and RBC.

The EtAMA1 DNA vaccine enhanced the production of serum specific IgY and chicken IFN-γ levels. These results suggested that the DNA vaccine used in the present study induced immunity against coccidiosis by enhancing humoral and cell-mediated immunity. In a similar study by

Hoan *et al.* (2014), DNA vaccines against coccidiosis stimulated the production of serum EbAMA1 specific IgG levels. Birds have been reported to produce parasite-specific antibodies in circulation and across mucosal surfaces in response to coccidial infection, but the role of humoral immune responses in conferring protective immunity against coccidiosis is unclear (Lillehoj and Trout, 1996; Yun *et al.,* 2000). However, studies by Belli *et al*.(2004) and Constantinoiu *et al*.(2008) demonstrated that antibodies play an important role in immunity against coccidiosis. Lillehoj and Lillehoj (2000) suggested that antibodies prevent translocation of sporozoites and merozoites across mucousal membranes, and this might be important in neutralizing extracellular stages of *Eimeria*. In this study, significant IgY responses were observed at 7 days post-vaccination which continued to increase when boosted and then followed by a sharp decrease post-challenge. The antibody neutralization might be due to antibody-antigen reaction as suggested by Lillehoj and Lillehoj (2000). Similar patterns of IgY response have been reported in chickens following immunization and challenge with *Eimeria* oocysts (Hoan *et al.,* 2014; Kundu *et al.,* 2017) contrarily to Rafiqi *et al.* (2018) who reported a continued increase in IgY post-challenge. However, the increase in IgY response may not be effective in relation to the control of *Eimeria* species as humoral immune responses also occur following coccidial infection, but antibodies play a minor role in resistance and immunity to coccidia (Long and Millard, 1979). The serum level of interferon-ϒ (IFN-ϒ) showed no significant ($P > 0.05$) difference among the groups of chickens at day 14 of age prior to vaccination. At days 21 and 28, the serum level of IFN-ϒ significantly ($P < 0.05$) increased in G3 and G4 chickens compared to G1 and G2 chickens. Furthermore, significant ($P < 0.05$) increase in serum level of IFN- ϒ was observed at day 35 in G3 and G4 chickens compared to chickens in G2 following challenge. This resultsrevealed that IFN-γ levels were significantly higher ($P < 0.05$) in vaccinated groups than unvaccinated groups. Increase serum level of IFN-γ has been reported in chickens' immunized using rEtSO7 (Rafiqi *et al.*, 2018). The higher oocyst reduction observed in vaccinated birds in this study might be due to the increase in IFN-γ as suggested by Zhu *et al.* (2012) and Hoan *et al.* (2014). IFN-γ has also been reported to mediates nitric oxide production thus, contributes to intracellular parasite killing (Ovington *et al.,* 1995). However, high levels of nitric oxide are detrimental for the host since it causes tissue damage (Allen, 1997) and this could possibly contribute partly to the caecal mucosal sloughing observed in vaccinated birds in this study. Thefindings of the present study indicate that the EtAMA1 DNA vaccine induced significant immune response involving cytokine and resulted in considerable protectionin the immunized chickens following experimental heterologous*Eimeria tenella* infection.

Lastly, challenge infection caused severe intestinal lesions such as necrotic mucosal glands and desquamation of the epithelium which were pinpointing towards the active disease faced by the positive control group in this study.Subramanian *et al.* (2008) observed only a marginal reduction in the lesion score in vaccinated birds while evaluating potential of EtMIC1 invasion as a candidate sub-unit vaccine for poultry. In contrast, all immunized chickens produced significantly less lesion score as compared to non-immunized challenged chickens in this study. Also, an observation contrary to Song *et al*. (2009) who observed mean lesion score 1.57 ± 0.77 after homologue challenge following immunization with pcDNA-TA4-IL-2 is that, while they carry out primary immunization at 7 days and booster immunization at 14 days old, in the current study, the primary immunization was at 14 days old and boosted at 21 days, to exclude the influence of the maternal immune response on the outcome of the immunization (Min *et al*., 2002). An interval of seven days between last boost and the euthanasia of the chicken was instituted. At the moment, a number of procedures are applied for coccidial challenge experiment including the interval of different days between the vaccinations and between last vaccinations to euthanasia. Many researchers used the challenge infection time 1 week post last immunization and obtained satisfactory results (Song *et al*., 2000, Min *et al*., 2002, Xu *et al*., 2008; Ali *et al*., 2014) as carried out in this research. However, intestinal lesions in coccidiosis are caused, in part, because of the host response to the infection. Moreover, the strain of coccidial oocyst used and the dose of the oocyst also determine the severity of lesions (Allen *et al*., 2004).

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

From this study, the following conclusions were made;

i. Vaccination using EtAMA1 DNA vaccine decreased the degree of clinical signs and improved the performance of chickens challenged with *Eimeria tenella* as they were very severe in G2, mild in G3 and G4.

ii. There were significantly ($P<0.05$) higher weight gains in vaccinated challenged chickens G3 and G4 when compared to unvaccinated challenged chickens post-challenge.

iii. Feed conversion ratio was significantly ($P>0.05$) higher in unvaccinated challenged chickens G2 than in vaccinated challenged chickens G3 andG4.

iv. The survival rate was 100% for chickens in G1 (unvaccinated unchallenged), G2 (unvaccinated challenged), G3 (vaccinated + Local isolate) and G4 (vaccinated + Houghton strain).

v. The oocyst decrease ratios and anticoccidial indices were significantly higher ($P<0.05$) in vaccinated challenged chickens G3 and G4 compared to unvaccinated challenged chickens 7 days post-challenge.

vi. Packed cell volume, haemoglobin concentration, red blood cells and total protein were significantly lower ($P<0.05$) in unvaccinated challenged chickens compared to vaccinated challenged chickens, G3 and G4.

vii. Total white blood cell, heterophil count and heterophil/lymphocyte ratio were significantly higher ($P<0.05$) in unvaccinated challenged chickens, G2 compared to vaccinated challenged chickens, G3and G4.

viii. A significantly higher ($P<0.05$) serum level of IgY was recorded in G3 and G4 7 days after primary vaccination, reaching a peak of and respectively, seven days post booster vaccination and lowered to and respectively seven days post challenge.

ix. Significantly higher ($P<0.05$) serum level of IFN- ϒ was recorded at day 35 of age in vaccinated challenged groups, G3 and G4 compared to unvaccinated challenge group,G2.

x. Mean caecal weight of vaccinated challenged chickens, G3 and G4 was significantly ($P<0.05$) greater in unvaccinated challenged chickens, G2.

xi. Gross lesions score was significantly ($P<0.05$) less in vaccinated challenged chickens, G4 and G3 with score of 2 (moderate) compared to unvaccinated challenged chickens (G2) with score of 4 (very severe).

xii. Histopathological changes in the caeca of unvaccinated challenged chickens, G2 showed desquamation of the epithelium and necrotic mucosal glands in the caecum, while those of vaccinated challenged chickens, G3 and G4 had intact mucosal glands and tissue architecture.

xiii. The findings of the present study indicate that the EtAMA1 DNA vaccine induced significant immune response involving cytokine and resulted in considerable protection in the immunized chickens following experimental heterologous *Eimeria tenella* infection.

6.2 Recommendations

i. Field trials to assess the efficacy of EtAMA1 DNA vaccine should be carried out.

ii. Experimental studies with EtAMA1 DNA vaccine involving differentdose, route and age ofvaccination should be performed.

6.3 Limitations of the Study

The major limitation in this study (that could be addressed in future research) is that, a Miniprep kit was used to produce DNA for the vaccination which gave an inadequate quantity to vaccinate the initial sample size of 20 birds per group, leading to a sample size reduction of 20 to 10 chickens

6.4 Contribution to Knowledge

The findings of the present study indicate that:

1. EtAMA1 DNA vaccine elicited protective immunity involving cytokine in broiler chickens following experimental infection with heterologous *Eimeria tenella*

2. Caecal lesion score caused by *Eimeria tenella* infection is invariably proportional to caecal weight

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APPENDICES

Appendix 1: Mean body weights of broiler chickens immunized with *Eimeria tenella* apical membrane antigen-1(EtAMA1) DNA vaccine and challenged with Local isolate or Houghton strain of *Eimeria tenella*

		Age (in days)			
		14	21	28	35
Group	Treatment	Mean body weight (g)			
G1	Unvaccinated unchallenged	615.00 ± 21.15	1,115.00 ± 23.63	1,590.00 ± 34.80	2,230.00 ± 24.94
G2	Unvaccinated challenged	585.00 ± 21.15	1,090.00 ± 31.45	1,520.00 ± 18.56	2,010.00 ± 22.11
G3	Vaccinated + Local isolate	590.00 ± 12.47	1,035.00 ± 16.75	1,530.00 ± 32.66	2,090.00 ± 22.11
G4	Vaccinated + Houghton strain	610.00 ± 6.67	1,070.00 ± 26.03	1,560.00 ± 33.17	2,125.00 ± 47.87

Appendix2: Mean packed cell volume of broiler chickens immunized with *Eimeria tenella* apical membrane antigen-1(EtAMA1) DNA vaccine and challenged with Local isolate or Houghton strain of *Eimeria tenella*.

		Pre-challenge	7 days post-challenge	% Change
Group	Treatment	Mean packed cell volume (%)		
G1	Unvaccinated unchallenged	28.40 ± 0.51	29.00 ± 0.84	+2.1
G2	Unvaccinated challenged	29.40 ± 0.68	19.80 ± 0.66	-32.7
G3	Vaccinated + Local isolate	29.20 ± 0.73	28.40 ± 1.08	-2.7
G4	Vaccinated + Houghton strain	28.80 ± 0.86	28.00 ± 0.84	-2.8

Appendix 3: Mean haemoglobin concentration of broiler chickens immunized with *Eimeria tenella* apical membrane antigen-1(EtAMA1) DNA vaccine and challenged with Local isolate or Houghton strain of *Eimeria tenella*.

		Pre-challenge	7 days post-challenge	% Change
Group	Treatment	Mean haemoglobin concentration (g/dL)		
G1	Unvaccinated unchallenged	9.46 ± 0.18	9.68 ± 0.27	2.32
G2	Unvaccinated challenged	9.64 ± 0.19	6.60 ± 0.22	-31.53
G3	Vaccinated + Local isolate	9.56 ± 0.24	9.42 ± 0.21	-1.46
G4	Vaccinated + Houghton strain	9.60 ± 0.28	9.58 ± 0.45	-0.20

Appendix 4: Mean total protein concentration of broiler chickens immunized with *Eimeria tenella* apical membrane antigen-1(EtAMA1) DNA vaccine and challenged withLocal isolate or Houghton strain of *Eimeria tenella*.

		Pre-challenge	7 days post-challenge	% Change
Group	Treatment	Mean total protein concentration (g/dL)		
G1	Unvaccinated unchallenged	4.48 ± 0.10	4.54 ± 0.09	1.34
G2	Unvaccinated challenged	4.34 ± 0.10	3.44 ± 0.15	-20.74
G3	Vaccinated + Local isolate	4.40 ± 0.16	4.42 ± 0.11	0.45
G4	Vaccinated + Houghton strain	4.54 ± 0.28	4.30 ± 0.15	-5.28

Appendix 5: Mean total red blood cell count of broiler chickens immunized with *Eimeria tenella* apical membrane antigen-1(EtAMA1) DNA vaccine and challenged with Local isolate or Houghton strain of *Eimeria tenella*.

		Pre-challenge	7 days post-challenge	% Change
Group	Treatment	Mean total red blood cell count (× 10^6/μL)		
G1	Unvaccinated unchallenged	4.48 ± 0.10	4.62 ± 0.14	3.13
G2	Unvaccinated challenged	4.67 ± 0.06	3.32 ± 0.13	-28.91
G3	Vaccinated + Local isolate	4.66 ± 0.13	4.5 ± 0.11	-3.43
G4	Vaccinated + Houghton strain	4.50 ± 0.07	4.48 ± 0.16	-0.44

Appendix 6: Mean total white blood cell count of broiler chickens immunized with *Eimeria tenella* apical membrane antigen-1(EtAMA1) DNA vaccine and challenged withLocal isolate or Houghton strain of *Eimeria tenella*.

		Pre-challenge	7 days post-challenge	% Change
Group	Treatment	Mean total white blood cell count ($\times10^3/\mu L$)		
G1	Unvaccinated unchallenged	20.66 ± 0.25	20.88 ± 0.21	+1.1
G2	Unvaccinated challenged	20.66 ± 0.31	22.22 ± 0.68	+7.0
G3	Vaccinated + Local isolate	20.80 ± 0.22	21.00 ± 0.47	+0.1
G4	Vaccinated + Houghton strain	20.62 ± 0.32	21.58 ± 0.49	+4.5

Appendix 7: Mean heterophil count of broiler chickens immunized with *Eimeria tenella* apical membrane antigen-1(EtAMA1) DNA vaccine and challenged withLocal isolate or Houghton strain of *Eimeria tenella*.

		Pre-challenge	7 days post-challenge	% Change
Group	Treatment	Mean heterophil count ($\times 10^3/\mu L$)		
G1	Non-vaccinated unchallenged	7.97 ± 0.24	7.90 ± 0.36	-0.90
G2	Non-vaccinated challenged	8.05 ± 0.58	8.92 ± 0.60	+10.80
G3	Vaccinated + Local isolate	7.66 ± 0.35	7.61 ± 0.34	-0.60
G4	Vaccinated + Houghton strain	7.59 ± 0.34	8.02 ± 0.27	+5.70

Appendix 8: Mean lymphocytes count of broiler chickens immunized with *Eimeria tenella* apical membrane antigen-1(EtAMA1) DNA vaccine and challenged with Local isolate or Houghton strain of *Eimeria tenella*.

		Pre-challenge	7 days post-challenge	% Change
Group	Treatment	Mean lymphocyte count ($\times 10^3/\mu L$)		
G1	Unvaccinated unchallenged	12.52 ± 0.36	12.81 ± 0.30	+2.32
G2	Unvaccinated challenged	12.32 ± 0.59	12.44 ± 0.53	+0.97
G3	Vaccinated + Local isolate	12.65 ± 0.38	13.14 ± 0.31	+3.87
G4	Vaccinated + Houghton strain	12.72 ± 0.57	13.44 ± 0.57	+5.66

Appendix 9: Mean heterophil/lymphocyte ratio of broiler chickens immunized with *Eimeria tenella* apical membrane antigen-1(EtAMA1) DNA vaccine and challenged with Local isolate or Houghton strain of *Eimeria tenella*.

		Pre-challenge	7 days post-challenge	% Change
Group	Treatment	Mean heterophil/lymphocyte ratio		
G1	Unvaccinated unchallenged	0.64 ± 0.04	0.62 ± 0.04	-3.13
G2	Unvaccinated challenged	0.67 ± 0.08	0.72 ± 0.06	+7.46
G3	Vaccinated + Local isolate	0.61 ± 0.04	0.58 ± 0.03	-4.92
G4	Vaccinated + Houghton strain	0.60 ± 0.05	0.60 ± 0.04	0.0

Appendix 10: Mean corpuscular volume of broiler chickens immunized with *Eimeria tenella* apical membrane antigen-1(EtAMA1) DNA vaccine and challenged with Local isolate or Houghton strain of *Eimeria tenella*.

		Pre-challenge	7 days post-challenge	% Change
Group	Treatment	Mean corpuscular volume (*f*L)		
G1	Unvaccinated unchallenged	63.51 ± 1.77	62.94 ± 2.31	-0.89
G2	Unvaccinated challenged	63.03 ± 1.73	59.70 ± 0.51	-5.28
G3	Vaccinated + Local isolate	62.72 ± 1.51	63.19 ± 2.37	-0.74
G4	Vaccinated + Houghton strain	64.19 ± 2.65	62.82 ± 3.05	-2.13

Appendix 11: Mean corpuscular haemoglobin of broiler chickens immunized with *Eimeria tenella* apical membrane antigen-1(EtAMA1) DNA vaccine and challenged with Local isolate or Houghton strain of *Eimeria tenella*.

		Pre-challenge	7 days post-challenge	% Change
Group	Treatment	Mean corpuscular haemoglobin (pg)		
G1	Unvaccinated unchallenged	21.17 ± 0.59	21.01 ± 0.74	0.76
G2	Unvaccinated challenged	21.01 ± 0.58	19.90 ± 0.21	-5.28
G3	Vaccinated + Local isolate	20.91 ± 0.50	21.06 ± 0.79	0.72
G4	Vaccinated + Houghton strain	21.40 ± 0.86	21.45 ± 1.08	0.23

Appendix 12: Mean serum immunoglobulin-Y level of broiler chickens immunized with *Eimeria tenella* apical membrane antigen-1(EtAMA1) DNA vaccine and challenged with Local isolate or Houghton strain of *Eimeria tenella*.

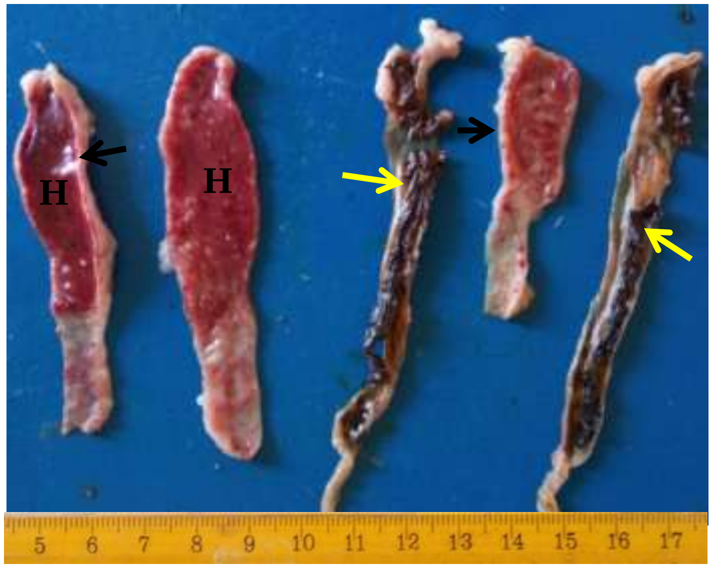
		Age (in days)			
		14	21	28	35
Group	Treatment	Mean serum IgY level (µg/mL)			
G1	Unvaccinated unchallenged	1.47 ± 0.04	1.44 ± 0.05	1.40 ± 0.05	1.23 ± 0.03
G2	Unvaccinated challenged	1.53 ± 0.05	1.45 ± 0.05	1.39 ± 0.06	1.05 ± 0.02
G3	Vaccinated + Local isolate	1.48 ± 0.08	6.08 ± 0.16	11.70 ± 0.23	9.16 ± 0.16
G4	Vaccinated + Houghton strain	1.43 ± 0.08	5.96 ± 0.09	11.64 ± 0.23	7.78 ± 0.18

Appendix 13: Mean serum interferon-ϒ level of broiler chickens immunized with *Eimeria tenella* apical membrane antigen-1(EtAMA1) DNA vaccine and challenged with Local isolate or Houghton strain of *Eimeria tenella*.

		Age (in days)			
		14	21	28	35
Group	Treatment	Mean serum interferon-ϒ level (pg/mL)			
G1	Unvaccinated unchallenged	1.61 ± 0.16	1.41 ± 0.21	1.50 ± 0.18	1.32 ± 0.19
G2	Unvaccinated challenged	1.37 ± 0.06	1.34 ± 0.19	1.34 ± 0.02	0.99 ± 0.04
G3	Vaccinated + Local isolate	1.36 ± 0.04	40.83 ± 0.50	62.60 ± 0.57	116.30 ± 0.28
G4	Vaccinated + Houghton strain	1.46 ± 0.17	38.85 ± 0.48	61.62 ± 0.50	115.90 ± 0.44

Appendix 14: Photograph of caeca of chickens in G1 (Unvaccinated unchallenged) and G2 (Unvaccinated challenged)

Photograph of caeca of chickens in G1 with no gross lesions. Score of 0



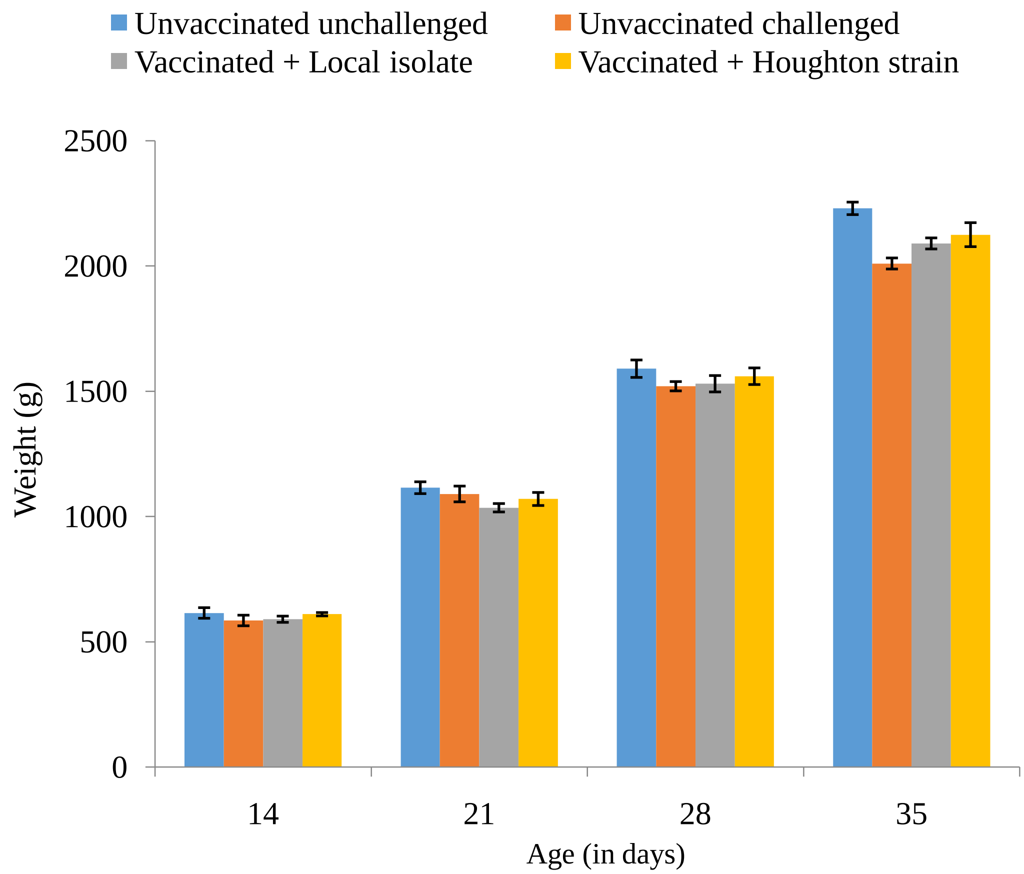
Photograph of caeca of chickens in G2 with haemorrhagic content (H), thickened walls (black arrows) and caecal cores (yellow arrows). Score of 3

Appendix 15: Photograph of caeca of chickens in G3 (Vaccinated challenged with Local isolate) and G4 (Vaccinated challenged with Houghton strain)

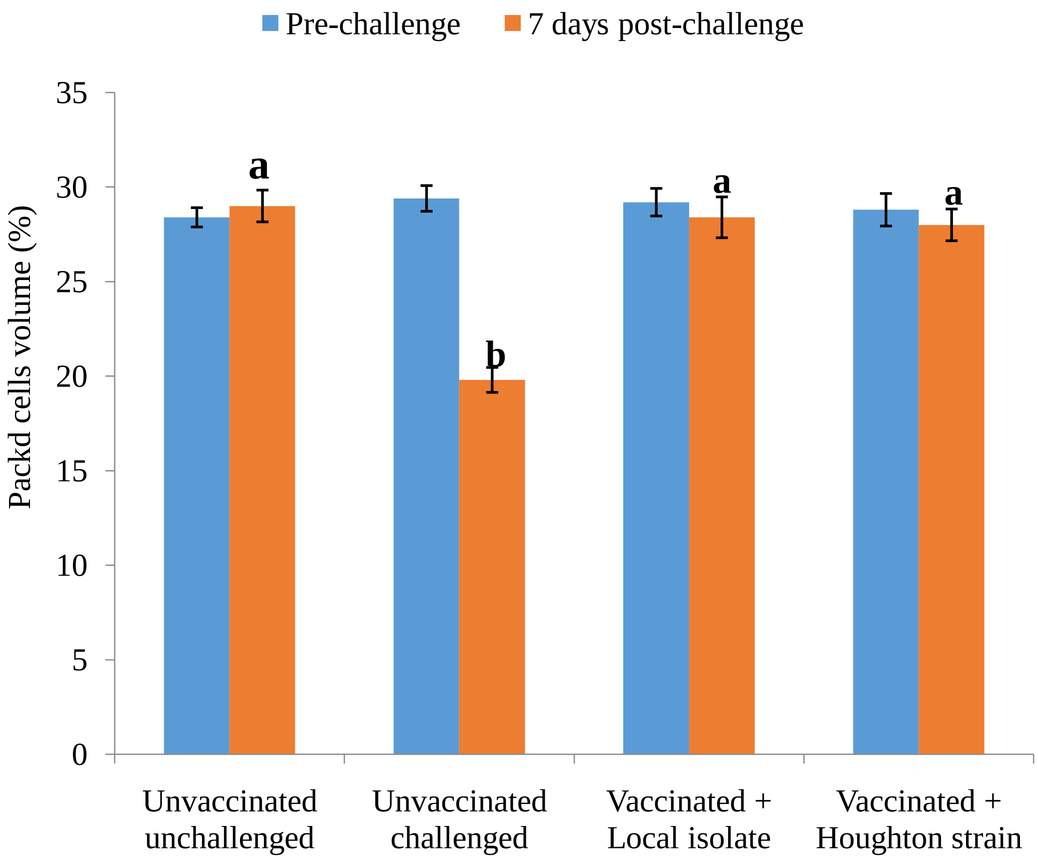
Photograph of caeca of chickens in G3 with petechiations (yellow arrows) and thickened walls (black arrows). Score of 2

Photograph of caeca of chickens in G4 with few petechiations (yellow arrows) and thickened walls. Score of 2

Appendix 16: Mean body weights of broiler chickens immunized with *Eimeria tenella* apical membrane antigen-1 (EtAMA1) DNA vaccine and challenged with local isolate or Houghton strain of *Eimeria tenella*. [a,b,c]Means with different superscript letters are significantly ($P < 0.05$) different

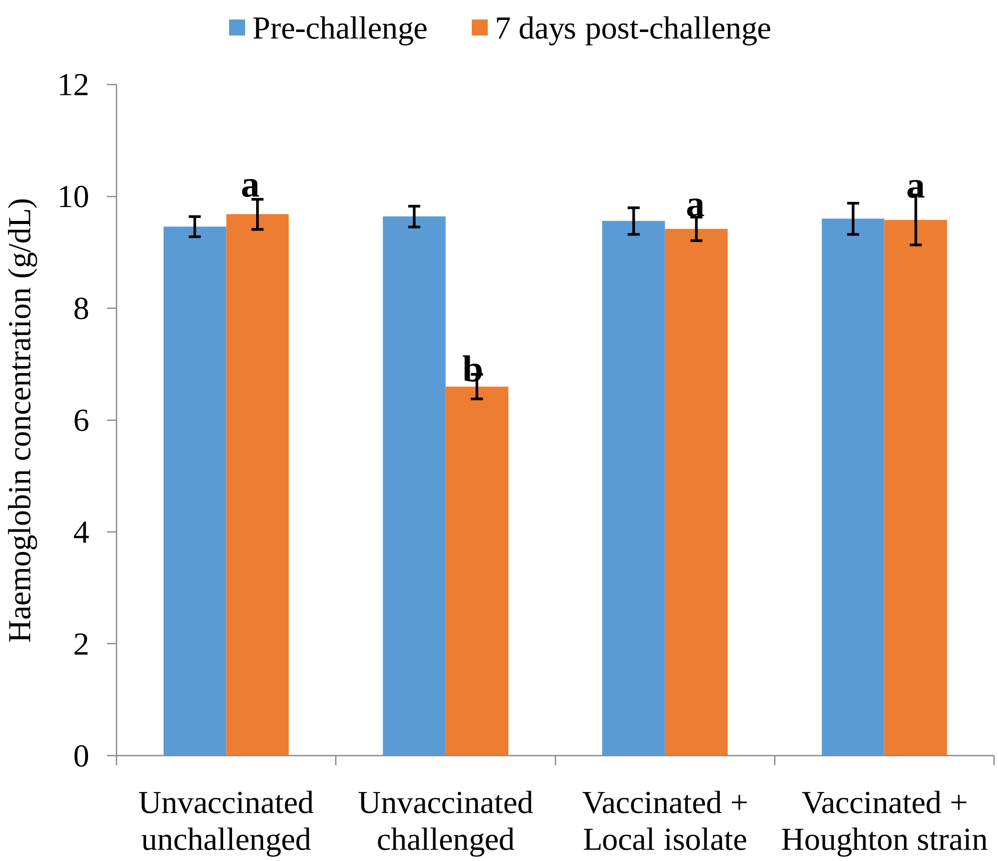


Appendix 17: Packed cell volume of broiler chickens immunized with *Eimeria tenella* apical membrane antigen-1(EtAMA1) DNA vaccine and challenged with Local isolate or Houghton strain of *Eimeria tenella*.[a,b]Means with different superscript letters are significantly (P < 0.05) different

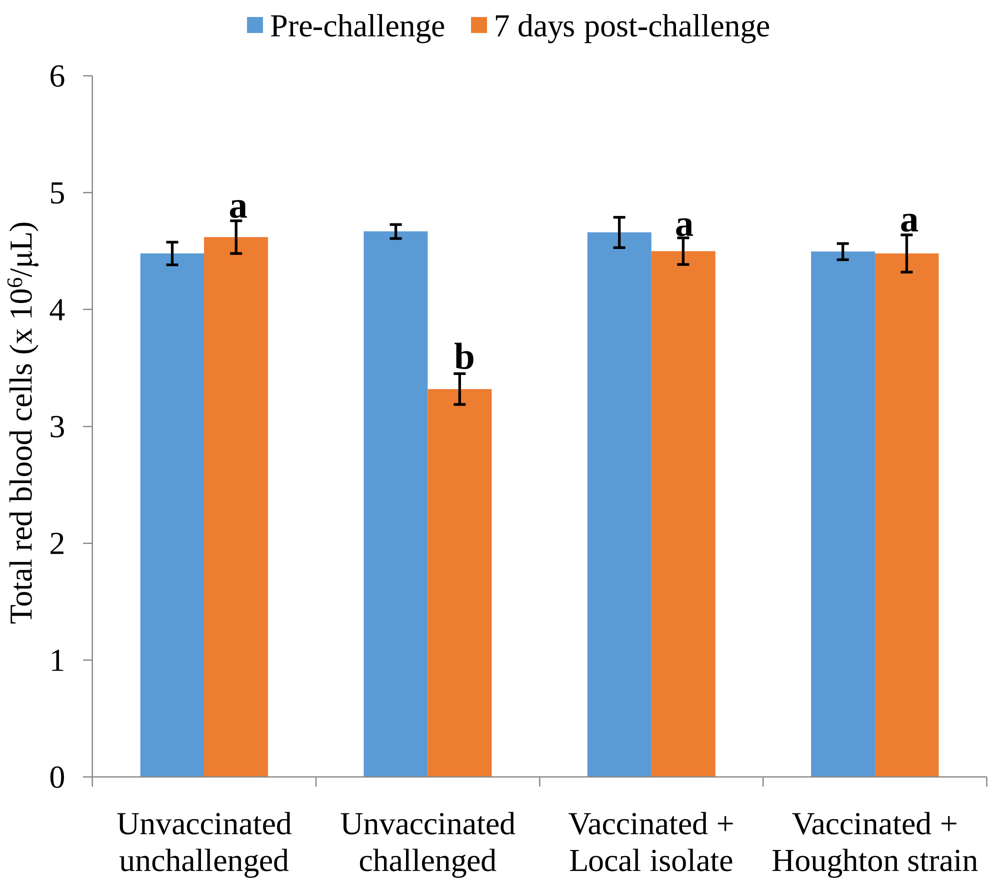


Appendix 18: Haemoglobin concentration of broiler chickens immunized with *Eimeria tenella* apical membrane antigen-1(EtAMA1) DNA vaccine and challenged with Local

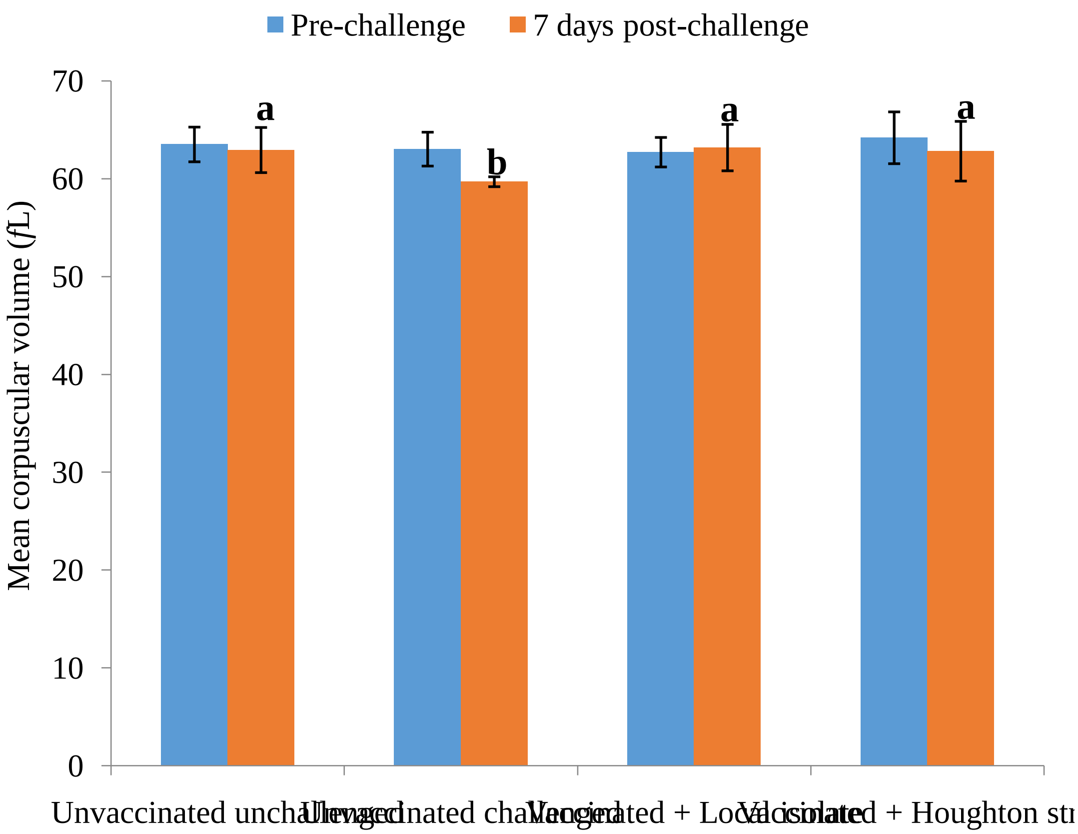
isolate or Houghton strain of *Eimeria tenella*.[a,b]Means with different superscript letters are significantly ($P < 0.05$) different



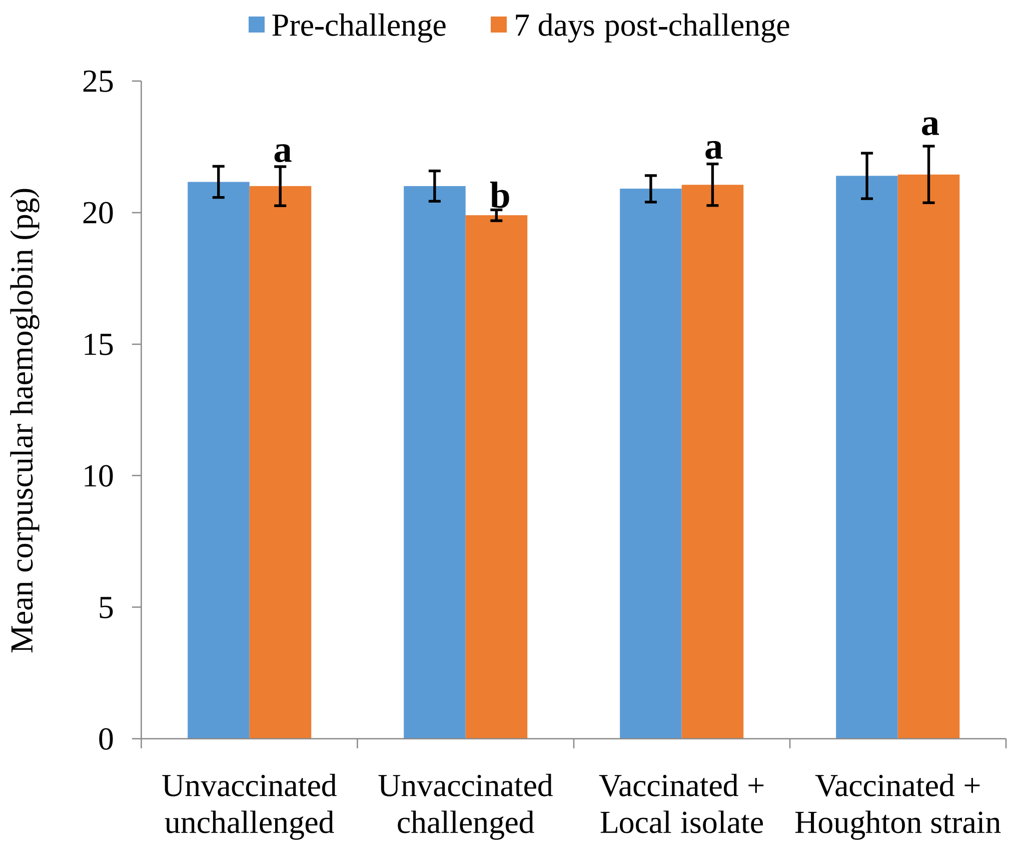
Appendix 19: Total red blood cell count of broiler chickens immunized with *Eimeria tenella* apical membrane antigen-1(EtAMA1) DNA vaccine and challenged with Local isolate or Houghton strain of *Eimeria tenella*.[a,b]Means with different superscript letters are significantly ($P < 0.05$) different



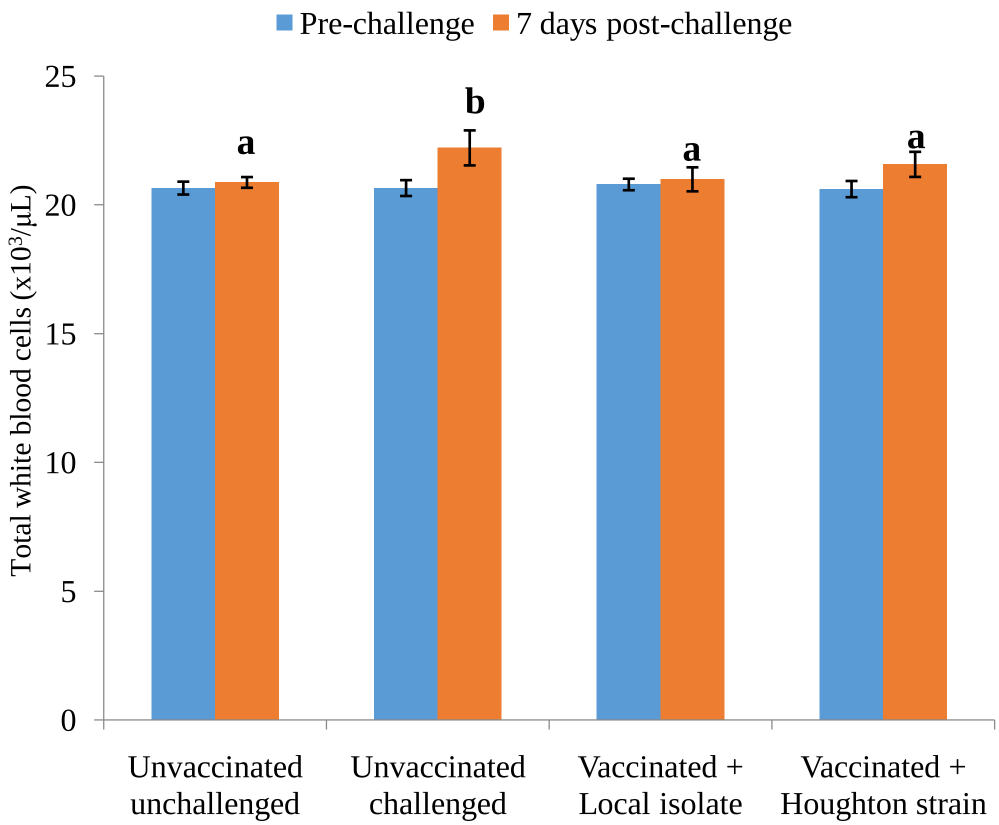
Appendix 20: Mean corpuscular volume of broiler chickens immunized with *Eimeria tenella* apical membrane antigen-1(EtAMA1) DNA vaccine and challenged with Local isolate or Houghton strain of *Eimeria tenella*.[a,b]Means with different superscript letters are significantly ($P < 0.05$) different



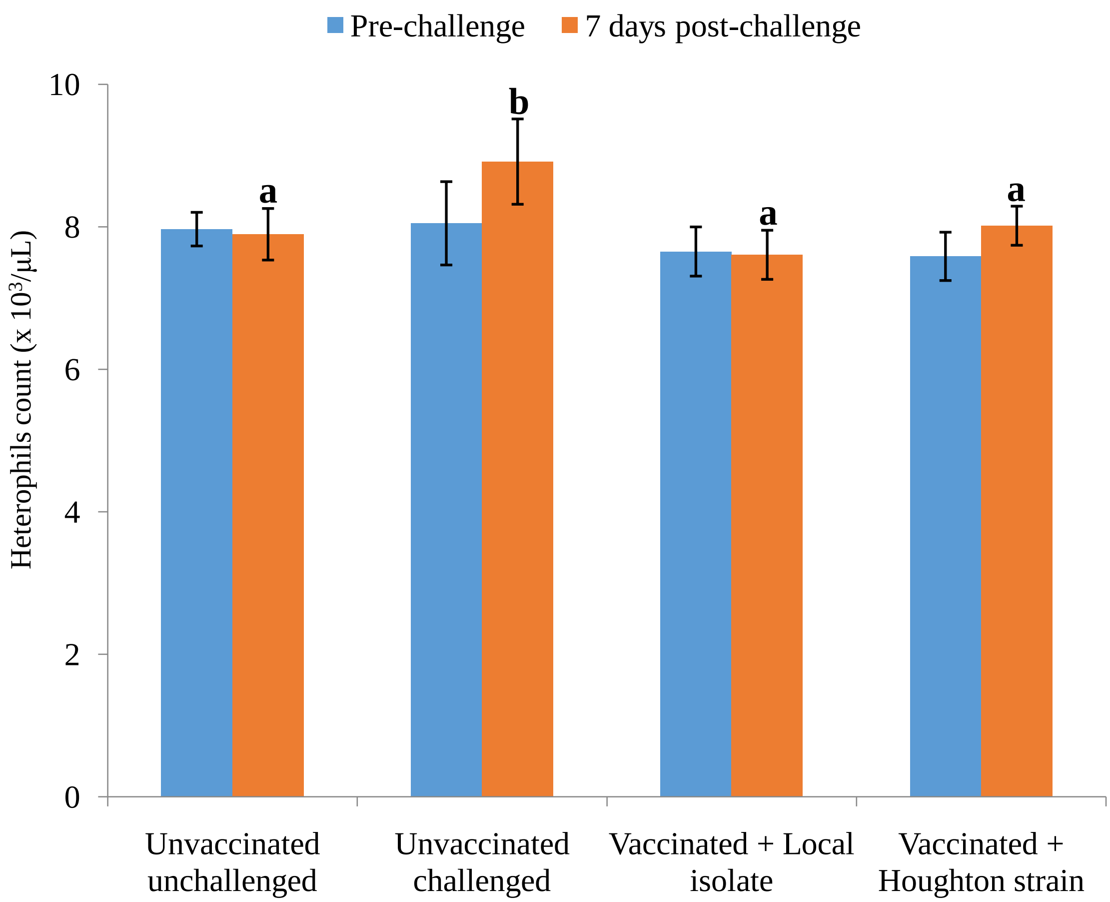
Appendix 21: Mean corpuscular haemoglobin of broiler chickens immunized with *Eimeria tenella* apical membrane antigen-1(EtAMA1) DNA vaccine and challenged with Local isolate or Houghton strain of *Eimeria tenella*.[a,b]Means with different superscript letters are significantly ($P < 0.05$) different



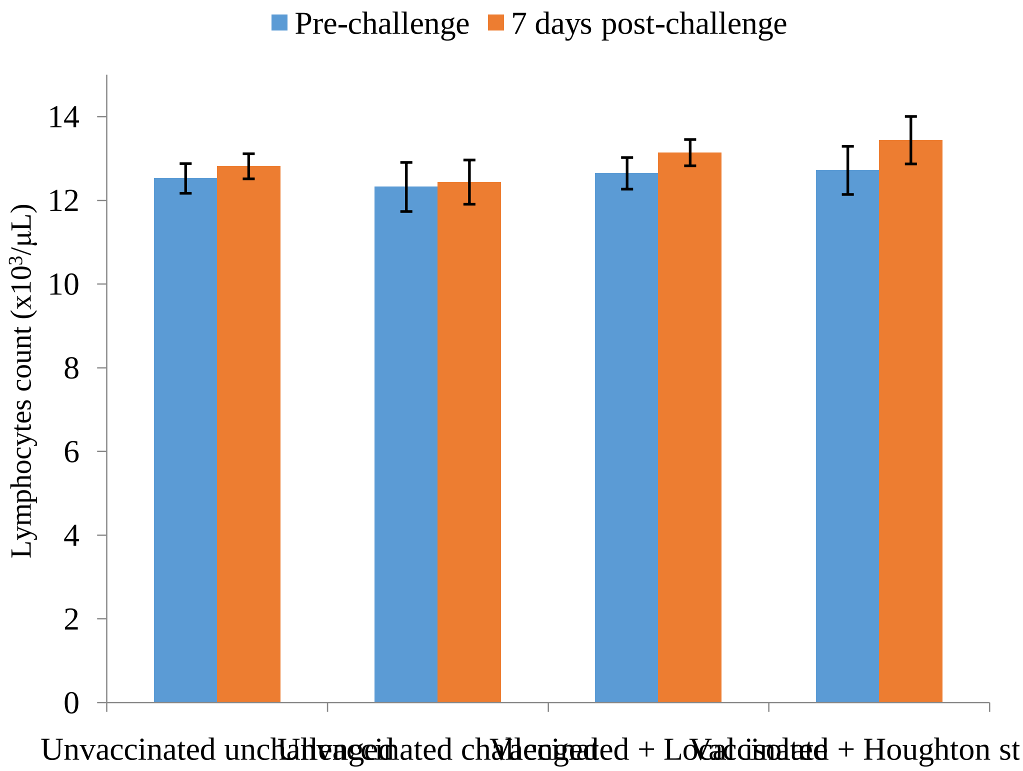
Appendix 22: Total white blood cell count of broiler chickens immunized with *Eimeria tenella* apical membrane antigen-1(EtAMA1) DNA vaccine and challenged with Local isolate or Houghton strain of *Eimeria tenella*.[a,b]Means with different superscript letters are significantly ($P < 0.05$) different



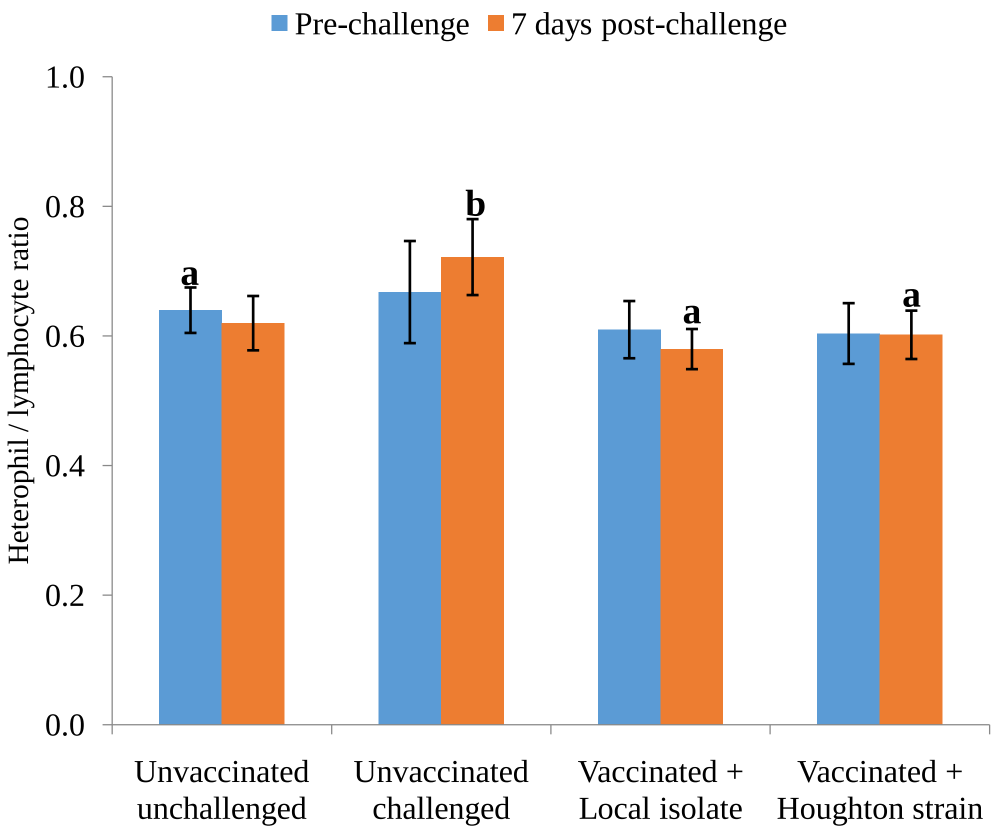
Appendix 23: Heterophil count of broiler chickens immunized with *Eimeria tenella* apical membrane antigen-1(EtAMA1) DNA vaccine and challenged with Local isolate or Houghton strain of *Eimeria tenella*.



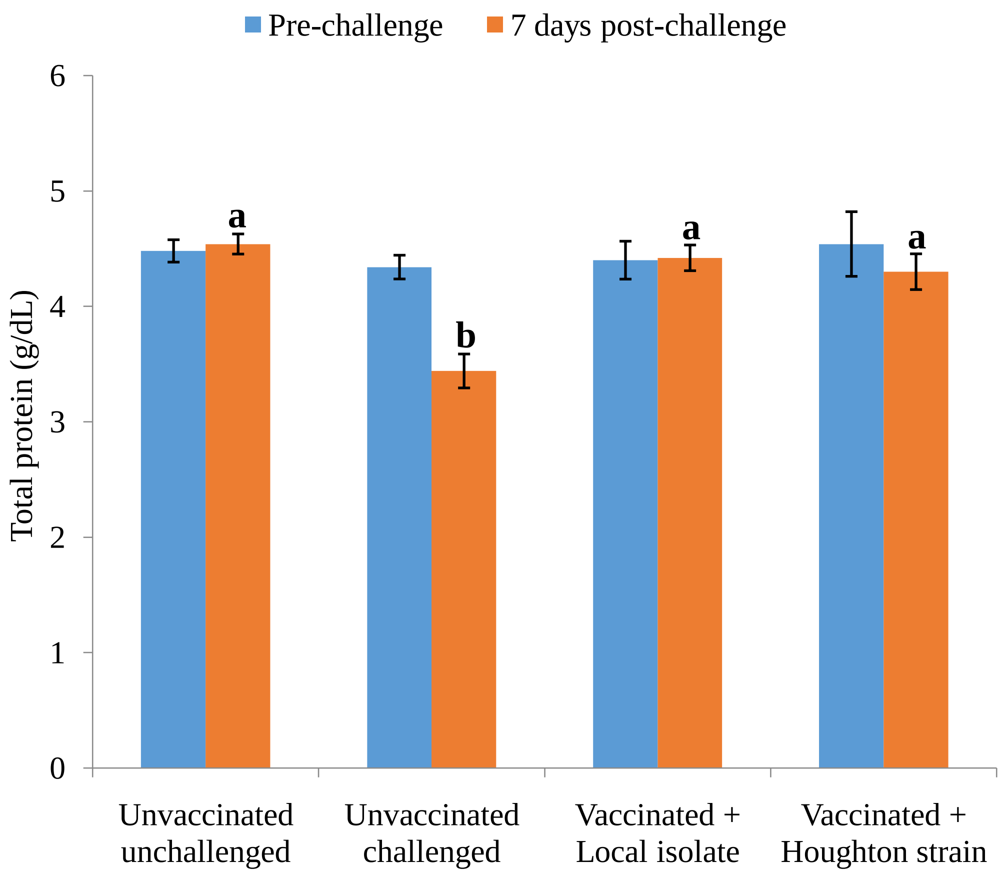
Appendix 24: Lymphocyte count of broiler chickens immunized with *Eimeria tenella* apical membrane antigen-1(EtAMA1) DNA vaccine and challenged with Local isolate or Houghton strain of *Eimeria tenella*.



Appendix 25: Heterophil/lymphocyte ratio of broiler chickens immunized with *Eimeria tenella* apical membrane antigen-1(EtAMA1) DNA vaccine and challenged with Local isolate or Houghton strain of *Eimeria tenella*.[a,b]Means with different superscript letters are significantly (P < 0.05) different



Appendix 26: Total protein concentration of broiler chickens immunized with *Eimeria tenella* apical membrane antigen-1(EtAMA1) DNA vaccine and challenged with Local isolate or Houghton strain of *Eimeria tenella*.[a,b]Means with different superscript letters are significantly (P < 0.05) different



Appendix 27: Ethical Clearance