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COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE

DEPARTMENT OF MICROBIOLOGY, PARASITOLOGY AND POULTRY HEALTH

MSc PROGRAM IN VETERINARY PARASITOLOGY

EFFICACY OF FORMALIN AND GAMMA RAY ATTENUATED COCCIDIAL VACCINES PRODUCED FROM LOCAL ISOLATES AGAINST CHALLENGE INFECTION IN BROILER CHICKEN

MSc THESIS

BY

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Efficacy of Formalin and Gamma Ray Attenuated Coccidial Vaccines Produced from Local Isolates Against Challenge Infection in Broiler Chicken

MSc Thesis Research

By

Melaku Yismaw

Submitted in Partial Fulfilment of the Requirements for the Degree of Master of Science (MSc) in Veterinary Parasitology

Advisors:

Professor Getachew Terefe (DVM, PhD)

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Addis Ababa University
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As members of the Examining Board of the final MSc open defense, we certify that we have read and evaluated the thesis prepared by Melaku Yismaw, entitled as: "**Efficacy of formalin and gamma ray attenuated coccidial vaccines produced from local isolates against challenge infection in broiler chicken**" and recommended that it be accepted as fulfilling the thesis requirement for the degree of Masters of Science in Veterinary Parasitology.

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STATEMENT OF DECLARATION

First, I declare that this thesis is my original work and that all sources of materials used for this thesis have been duly acknowledged. This thesis has been submitted in partial fulfillment of the requirements for an MSc degree at Addis Ababa University, College of Veterinary Medicine and Agriculture and is deposited at the College library to be made available to borrowers under the rules of the library. I solemnly declare that this thesis is not submitted to any other institution anywhere for award of any academic degree, diploma or certificate. Brief quotations from this thesis are allowed without special permission, provided that an accurate acknowledge of the source is made. Requests for permission for an extended quotation from or reproduction of this manuscript in whole or in part may be granted by the Dean of the College when, in his or her judgment, the proposed use of the material is in the interests of scholarship. In all other instances, however, permission must be obtained from the author.

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TABLE OF CONTENTS **PAGES**

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ABBREVIATIONS

ACI	Anticoccidial Index
BW	Body Weight
CVMA	College of Veterinary Medicine and Agriculture
DNA	Deoxyribonucleic Acid
GSR	Growth and Survival Rate
$K_2Cr_2O_7$	Potassium Dichromate
LS	Lesion Scores
NV-NC	Non-vaccinated Non-infected Control
NV-PC	Non-vaccinated Infected Control
OPG	Oocyst per Gram
PCH	Post-challenge
POAA	Percent Optimum Anticoccidial Activity
PIV	Post-initial Vaccination
RLS	Reduction of Lesion Score
RNA	Ribonucleic Acid
ROP	Relative Oocyst Production

LIST OF TABLES **PAGES**

LIST OF FIGURES **PAGES**

LIST OF ANNEXES **PAGES**

ABSTRACT

Poultry coccidiosis, a major intestinal parasitic disease caused by Eimeria, has developed drug resistance, but vaccine use in Ethiopia remains limited. This study aimed to evaluate safety and efficacy of formalin and gamma ray attenuated coccidial vaccines produced from local isolates against challenge infection in broiler chickens. Oocysts were purified from infected poultry farms for propagation in chickens and purified, and used to develop attenuated vaccines via formalin (1.2%) and gamma ray (150 Gray). The acute vaccine safety test of both vaccines showed mild clinical coccidiosis, indicating the vaccines were safe. In a randomized control trial, 150 day-old Cobb500 chickens were divided into ten groups of 15 chickens (five in three replicates). The chickens received either formalin (FR) or gamma ray (GR) vaccines at two doses (500 and 1000) with challenge and non-challenge groups. Vaccines were given orally on days 4 and 11 at the same dose, and challenged with 1×10^5 virulent oocysts on 21 day of age. The control group included non-vaccinated challenged control (NV-PC) and non-vaccinated non-challenged (NV-NC). Clinical signs, oocyst output, weight gain, survival rate and intestinal lesions were monitored. Vaccine efficacy was evaluated using anticoccidial index (ACI), relative oocyst production (ROP), percent optimum anticoccidial activity (POAA) and reduction of lesion score (RLS). The result revealed that both vaccine types induced mild infection after vaccination. While post-challenge, vaccinated chicks showed mild clinical signs, reduced oocyst output, higher weight gain, 100% survival rate and reduced lesion score compared to NV-PC group. Among vaccinated groups, the one that received 1000 γ-radiated oocysts (GR_{1000}) had the lowest oocyst output and higher weight gain than others ($p<0.05$). Vaccine efficacy index showed that GR_{1000} group had the highest efficacy with higher ACI (>180), low ROP (<15%), high POAA and RLS (>50%), while GR_{500} also performed well. In contrast, groups that received oocyst attenuated using formalin (FR_{1000} and FR_{500}) exhibited limited or no protective efficacy. In conclusion, γ-ray attenuated sporulated oocysts can be a good candidate for producing an effective anti-coccidial vaccine from local strains of the parasite. Further study is required to test different doses, routes of administration and the shelf life of the product under different temperatures.

Keywords: *Attenuation, Chicken, Coccidia, Efficacy, Formalin, Gamma ray*

1. INTRODUCTION

All domestic birds, including geese, ostriches, ducks, turkeys, chickens, peacocks, and pheasants, are referred to as poultry. The most significant are chickens (*Gallus domesticus*) (Shakoor *et al*., 2021). They are primarily kept to produce meat and eggs for human consumption and manure for crops (Adem and Ame, 2023). The productivity of chickens is affected by several factors, including handling, housing, predators, and poultry rearing, in addition to diseases (Quiroz-Castañeda, 2018).

Coccidiosis, caused by protozoan parasites of numerous Eimeria species, has long been recognized as a devastating disease that causes severe economic consequences worldwide (Juárez-Estrada *et al*., 2023). The prevalence of coccidial infection varies between 10% and 90% worldwide, whereas in Ethiopia from 22.9% to 71.7% in intensive chicken farms, and affects mostly young chickens in all production systems **(**Engidaw and Getachew, 2018). Both direct and indirect losses cause profit losses in large and small farms of 8.4% and 11.86%, respectively (Tirfie and Lulie, 2024). According to Khater *et al*. (2020), Eimeria species cause intestinal wall damage by penetrating the host's intestinal cells at several places. This leads to poor absorption, decreased weight gain, dysentery, dehydration, and death (Cai *et al*., 2023).

Today, the most popular preventive methods are based on chemotherapy; using anticoccidial drugs, along with hygienic measures and improved farm management (Pal *et al*., 2024). However, cross and multiple-resistance to existing anticoccidial drugs is becoming a challenge (Flores *et al*., 2022), and drug residues in meat and eggs are also posing public health concerns (Tang *et al*., 2018). Such challenges have prompted the search for anticoccidial vaccines as crucial tools in the fight against coccidiosis (Cai *et al*., 2022). Different types of vaccines have been made by using virulent or attenuated strains (Blake *et al*., 2017) and using recombinant deoxyribonucleic acid (DNA) technology, such as subunit vaccines, DNA vaccines, and vector vaccines (Kota *et al*., 2017). While each type of vaccine candidate has its own advantage and limitations, attenuated vaccines are said to provide superior protection compared to other types of vaccines (Falsafi *et al*., 2023).

Several methods of attenuation have been used, including selection for serial passage in chicken embryos and chickens (precociousness), and chemical, heat, or irradiation treatment to decrease the pathogenicity of the parasite (Mesa-Pineda *et al*., 2021) without affecting their immunogenicity. Sporulated oocysts attenuated by using chemical and physical (use of gamma rays) methods are said to be safe and effective means to minimize their pathogenicity. With this technique, the protein coat structure is maintained, which acts as an immunogen to induce protective immunity in chickens (Bahrami and Bahrami, 2006; Toka and Geinoro, 2020; Djemai *et al*., 2023). In vaccinated chickens, a challenge infection results in higher feed conversion ratios, less oocyst shedding in feces, good body weight gains, less severe intestinal lesions, and higher survival rates than in the control group (Zaheer *et al*., 2022).

Studies using formalin attenuated *E. tenella* oocyst have reported on reduced cecum lesions score (Setyowati *et al*., 2019), decreased oocyst production without challenge infection (Anggraini *et al*., 2021), and reduced cecal lesion scores after challenge infection of vaccinated chicken (Armiani *et al*., 2019). Gamma ray irradiated *E. maxima, E. acervulina,* and *E. tenella* oocysts have also been reported to induce protective immunity in chickens (Fetterer *et al*., 2014). Similarly, a low energy electron irradiation has also been used to protect chickens against cecal coccidiosis (Thabet *et al*., 2019). Live attenuated or virulent multivalent anticoccidial vaccines such as Evalon®, Coccivac® and Evant® have long been on the global market (Attree *et al*., 2021).

In Ethiopia, poultry production is becoming popular in both rural and urban areas. According to the agricultural sample survey data from the Central Statistical Service (CSA, 2021), the total poultry population in the country is estimated at about 57 million. Cognizant of its contribution to the livelihood of many families and its overall economic benefit, Ethiopia has given tremendous attention to promoting the poultry industry (Fekadu *et al*., 2022). However, poultry diseases such as coccidiosis are responsible for significant reduction in poultry farm productivity. According to a study by Hailegebreal *et al*. (2022), among clinical cases of coccidiosis encountered in three farms, a case fatality rate of 46% was observed. Management of this parasitic problem is mainly based on the use of prophylactic or curative anticoccidial drugs. While in many parts of the

world, vaccination is becoming an important control strategy for coccidiosis in broiler chickens, neither attenuated nor subunit or DNA anticoccidial vaccines is commercially available in Ethiopia. Among the main reasons for this are that such coccidial vaccines are expensive and the shelf life of live vaccines is limited prompting a search for local production of such vaccines.

The objectives of this research were:

General objective

- ✓ To produce an effective attenuated anti-coccidial vaccines against locally circulating isolates of Eimeria affecting commercial chickens.

Specific objectives

- ✓ To produce safe live attenuated anti-coccidial vaccines from local isolates of pathogenic Eimeria species
- ✓ To evaluate and compare the vaccinal efficacy of formalin and gamma radiation attenuated sporulated oocysts against challenge infection in chickens

2. LITERATURE REVIEW

2.1. Poultry Coccidiosis

Coccidiosis is a common and economically devastating intracellular parasitic disease of the poultry industry worldwide caused by several species of Eimeria protozoan within the intestine (Chere *et al*., 2022; Pal *et al*., 2024). The disease is characterized by paleness of the comb and wattle, bloody diarrhoea, depression, ruffled feathers, low growth and production (Adem and Ame, 2023) with a high rate of mortality and morbidity (Khater *et al*., 2020). The current approaches to treating coccidiosis are anticoccidial drugs, live vaccinations and strict management practices (Zhao *et al*., 2024).

2.1.1. Etiology

Poultry coccidiosis is an obligate intracellular intestinal parasitic infection caused by the Apicomplexan protozoan belonging to the subclass Coccidia, family Eimeriidae, and genus Eimeria (Cunha *et al*., 2020). In terms of economics, the seven most significant and unique species of the genus Eimeria invade the intestinal or cecal epithelial cells. These species include *E. acervulina, E. brunetti, E. maxima, E. mitis, E. praecox, E. necatrix,* and *E. tenella* (Shakoor *et al*., 2021). Two additional species, *E. hagani* and *E. mivati,* have been described, but their validity is not yet accepted. The latter taxon may be a variant of *E. mitis,* considered to be a *nomen dubium* (Bangoura and Daugschies, 2018).

2.1.2. Epidemiology

Poultry coccidiosis is a worldwide disease, it occurs throughout the year but endemic in most tropical and subtropical climates, where management and ecological conditions allow the causal agent to survive and propagate throughout the year (Abdisa *et al*., 2017). The prevalence of coccidiosis in chickens has been reported in several countries, namely Iran, Egypt, Ethiopia, South Africa, Nepal, Korea, and Nigeria (Das, 2021). All seven Eimeria species in Ethiopia were molecularly detected in small and large-scale broiler farms (Chere *et al*., 2022) and village chickens (Luu *et al*., 2013).

Key contributors to the onset of coccidiosis include the age and breed of the chicks, their health status, the management system in place, seasonal variations, and the level of biosecurity (Bharti *et al*., 2025). The age between 3 and 18 weeks is more susceptible; however, young chickens have higher mortality rates (El-shall *et al*., 2021). Oljira *et al.* (2012) stated that the Bovans (25.10%) were more susceptible than local breeds (12.41%) of chickens in and around Ambo town, Ethiopia.

According to Dakpogan and Salifou (2013) and Yu and Heo (2021), a high incidence of coccidiosis is usually observed in poultry managed under intensive management systems like deep litter due to the increased likelihood of high oocyst accumulation in the litters. Poor management practices that promote oocyst sporulation, such as wet litter, unsanitary feed and water troughs, insufficient ventilation, and high housing density, frequently exacerbate clinical symptoms (Blake *et al*., 2021; Ahmed *et al*., 2024). Coccidiosis was most prevalent in autumn at 45.12%, followed by summer (30.84%) and spring (23.81%), and was low in winter (20.29%) (Ahad *et al*., 2015). The development of drug-resistant strains is more common when continuously used anticoccidial drugs (Gao *et al*., 2024).

2.1.3. Life cycle and pathogenesis

When the host consumes the sporulated oocyst, four sporocysts, each containing two banana-shaped sporozoites, develop within its circumplasm, signaling the beginning of the infection (López-Osorio *et al*., 2020). The action of mechanical and chemical (i.e., trypsin, bile, and carbon dioxide) aids in the excystation of the oocysts within the intestinal lumen, and the eight sporozoites that are liberated enter the villous epithelial cells (Yu and Heo, 2021). Sporozoites of some species (*E. brunetti* and *E. praecox*) develop within cells at the site of penetration. Sporozoites of other species (*E. acervulina*, *E. maxima*, *E. necatrix*, and *E. tenella*) are transported to other sites, viz. the crypt epithelium, where they develop as trophozoites (Tewari and Maharana, 2011). The host cell is ruptured, and new cells are invaded by a high number of liberated merozoites. Male and female gametocytes can be produced from these merozoites. After fertilization, the oocysts burst open the host's enterocytes and leave with faeces (**Figure 1**) (Ahmad *et al*., 2016; Khater *et al*., 2020).

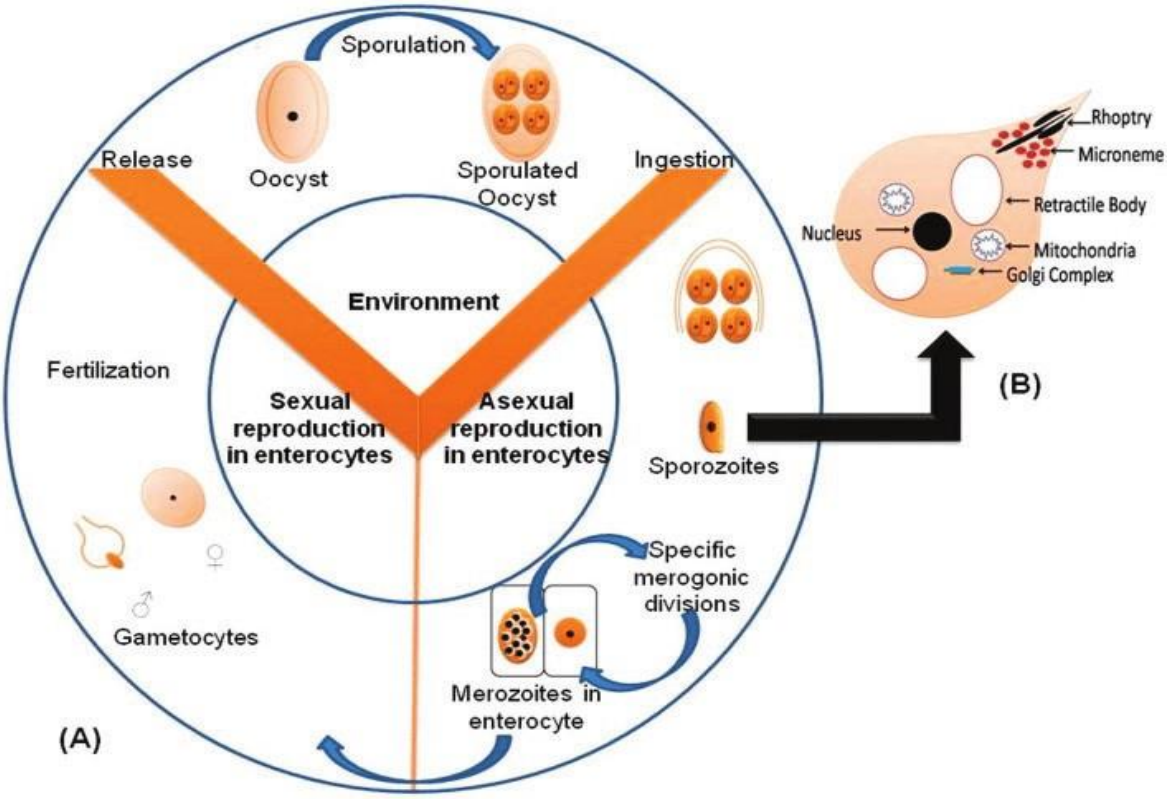

Figure 1: Life cycle of genus Eimeria

(A). Sexual and asexual stages of reproduction occur in epithelium cells, and oocyst formation occurs outside the chickens (B). Structure of Eimeria spp. sporozoite (Quiroz-Castañeda, 2018).

2.1.4. Clinical signs

Coccidial infection is classified into three types; infection by a sufficiently large number of coccidia produces clinical manifestations of the disease called coccidiosis, whereas subclinical infections are asymptomatic but cause adverse effects on performance. The mildest form of infection that causes no symptoms and no adverse effects on performance is called coccidiasis (Mesa-Pineda *et al*., 2021; Shakoor *et al*., 2021). The subclinical coccidiosis manifests mainly by poor weight gain and reduced efficiency of feed conversion rate, and gives rise to the highest proportion of the total economic loss (Engidaw and Getachew, 2018).

The intestinal epithelium and underlying mucosal connective tissue are destroyed in coccidiosis, resulting in clinical signs. When symptoms become more severe, it could also be accompanied by bleeding into the intestinal lumen (Yu and Heo, 2021). Accordingly, the clinical form of coccidiosis appears in weight loss, reduced feed intake, paleness, ruffled feathers, depression, pale-colored combs and wattles, huddling, closed eyes, diarrhea with bloody faeces, dehydration, and an increased number of mortalities that may accompany (Ahad *et al.*, 2023).

2.1.5. Post-mortem examination and lesion score

Each species of Eimeria has a different pathophysiology, with infections occurring in different parts of the intestine and leading to either hemorrhagic (*E. brunetti, E. necatrix*, and *E. tenella*) or malabsorptive (*E. acervulina*, *E. maxima, E. mitis*, and *E. praecox*) disease (Burrell *et al*., 2020). Mixed species infections are common, affecting different sections of chicken intestines (Andreopoulou *et al*., 2022). The parasite is site and host-specific and can be distinguished by the pathology it causes, the oocyst morphology, the minimum prepatent period, the minimum time needed for sporulation, and obvious macroscopic lesions (**Table 1**) (Liu *et al*., 2023; Gao *et al*., 2024). Based on macroscopically visible lesions in the gut caused by Eimeria spp., Johnson and Reid (1970) developed a post-mortem examination scoring system that ranks from 0 to +4 (i.e., 0 = no lesion, +1 = mild lesion, +2 = moderate lesion, +3 = severe lesion, and +4 = extremely severe lesion).

Table 1: Main characteristics of Eimeria species in the poultry

Eimeria species	Predilection site	Level of pathogenicity	Condition of the lesion
E. praecox	Duodenum, jejunum	Low	No lesion but slightly haemorrhagic, slight mucoid discharge on the intestinal surface of the duodenum.
E. acervulina	Duodenum, ileum	Medium	Haemorrhage streaks with whitish lesions, mucoid and whitish spots on the intestinal surface.
E. mitis	Ileum	Low	Limited enteritis causing loss of fluids
E. maxima	Duodenum, jejunum, ileum	Medium	Distended intestine with haemorrhage spots, mucoid discharge.
E. brunetti	Caeca rectum	High	Thin-walled intestine, mucoid on necrotic discharge, and distension of the intestine.
E. tenella	Caeca	High	Severe haemorrhage with white and red spots on the wall of the intestine.
E. necatrix	Jejunum, ileum, caeca	High	Severe haemorrhage with mucoid discharge, whitish and red spots on the wall of the intestine.

Source: (Nawarathne *et al*., 2021)

2.1.6. Diagnosis

The pathogenic species of Eimeria may generally be distinguished based on clinical signs, oocyst faecal examination, lesion gross appearance, oocyst morphology, and sporulation time (Bora *et al*., 2024). Molecular biological methods can also be used as a diagnostic tool. Previous methods for identifying various species relied on ribosomal DNA and ribonucleic acid (RNA) probes, as well as the isoenzyme patterns of oocysts. According to Rasheed (2016), the polymerase chain reaction-based assay is regarded as a quick and precise identification technique.

2.1.7. Prevention and control measures

Coccidia oocysts are omnipresent; their high spreading ability and high reproductive ability make it difficult to keep chickens free of coccidiosis (Nawarathne *et al*., 2021). The current approach to preventing and controlling coccidiosis is based on chemotherapy, using anticoccidial drugs and vaccines, along with biosecurity, hygienic practice and improved farm management (Pal *et al*., 2024).

Anticoccidial drugs are the most widely utilized global treatments for avian coccidiosis (Lee *et al*., 2022). Since 1948, chicken farming has used chemical prophylaxis to control coccidiosis (Attree *et al*., 2021). Anticoccidials could be classed into synthetic and ionophorous/polyether (El-Ghany, 2021). Synthetic drugs are chemically synthesized to prevent different biochemical pathways of the developing parasite metabolism (Chapman *et al*., 2010). Ionophores/polyether antibiotics are manufactured by the fermentation of Streptomyces spp. or Actinomadura spp. arrest the ion transport channels and hinder the osmotic balance of the coccidian species (Peek and Landman, 2011; Yu and Heo, 2021).

Globally, widespread and extended use of anticoccidial drugs has led to the development of resistance in chickens. Lan *et al*. (2017) reported severe resistance to toltrazuril, sulfonamides/trimethoprim, and amprolium. Ojimelukwe *et al*. (2018) documented resistance to toltrazuril in experimentally infected chickens. Flores *et al*. (2022) identified severe resistance to multiple coccidiostats, including clopidol, diclazuril, maduramicin, monensin, salinomycin, and toltrazuril using nine different field samples.

In an experimental study, Hunduma and Kebede (2016) showed that coccidian organisms were responsive to the coccidiostat sulfadimidine but resistant to amprolium treatment in chickens raised at the Agricultural Research Centre in Bishoftu, Ethiopia. Different anticoccidial drugs are alternated in single and/or shuttle programs to reduce the possibility of resistance. Unfortunately, according to Peek and Landman (2011), this hasn't resolved the issue of anticoccidial resistance. Fortunately, vaccination is an effective alternative to anticoccidial drugs for control because Eimeria infections result in strong, long-lasting protection (El-Ghany, 2021; Lee *et al*., 2022).

Herbal medicine explores the utilization of plants and their extracts in the treatment and prevention of coccidiosis (El-shall *et al*., 2021). Plants such as garlic (*Allium sativum*) and ginger (*Zingiber officinale*) (Ali *et al*., 2019), oregano and Citrus spp. (Gordillo *et al*., 2021) and neem (*Azadirachta indica*) (Onyiche *et al*., 2021). Challenges in using herbal medicines for poultry coccidiosis include standardization, composition and mechanism of action, effective delivery methods, optimal dosage identification, and regulatory issues like a lack of approval in some countries (Abad and Ghaniei, 2023).

2.1.8. Economic importance

The economic significance of coccidiosis in poultry has long been recognized. The most significant expenses include increased rates of morbidity and mortality, reduction in productivity, culling, drug and vaccine costs for prevention and control of coccidiosis (Blake *et al*., 2020; Nawarathne *et al*., 2021). Although coccidiosis causes economic losses worldwide, it is more prominent in developing countries, and the world losses up to 3 billion dollars. Only from broiler production does the poultry industry earn almost 40 to 42 billion dollars annually, but coccidiosis causes major losses that reach up to 2.4 billion dollars annually (Abbas *et al*., 2024). In Debre Zeit, Ethiopia, a study on poultry farms of different scales revealed that coccidiosis leads to profit losses of 8.4% in large farms and 11.86% in smaller ones (Dinka and Tolossa, 2012).

2.1.9. Status of poultry coccidiosis in Ethiopia

In Ethiopia, coccidiosis is a prevalent and economically important disease that affects chickens. The disease is widespread throughout the country and causes significant losses for the industry, especially for young birds in all production systems. An outbreak is often responsible for 80% of disease occurrences (Adem and Ame, 2023). The prevalence of coccidiosis has been reported different regions of the country. For instance, a study by Dinka and Tolossa (2012) in Debre Zeit (71.1%), Oljira *et al.* (2012) in and Around Ambo town (20.5%), Cheru *et al*. (2023) in East Gojjam Zone (10.2%), and Yayeh (2025) in Gondar with the prevalence of 22.4%.

2.2. Anticoccidial Vaccine

Vaccination is an effective and successful method against coccidiosis because vaccines provide a high level of protection against coccidiosis and replace the populations of *Eimeria*, which reduces the drug resistance problem in chickens and drug residues in animal-derived products (Khater *et al*., 2020; Tang *et al*., 2020; Gao *et al*., 2024). The potential areas for addressing chicken coccidiosis in commercial flocks need to be focused in terms of cost-effective vaccine production and commercialization on a practical basis (Zaheer *et al.*, 2022).

Eimeria spp. self-limiting, minimal antigen variation, and long-term immunity demonstrate the potential for developing a vaccine against coccidian infections (Shakoor *et al*., 2021). Chickens that have immunity to a single species of Eimeria usually have minimal or no cross-protection against other species (Gao *et al*., 2024). Primary infection is demonstrated by increased granulocytes, natural killer cell activity, and serum proteins, before an antigen-specific memory immune response is developed through lymphocytes and their secretions, in the form of antibodies and cytokines (Mesa-Pineda *et al*., 2021). B and T lymphocytes of the gut-associated lymphoid tissues may induce an active immunity in 3-4 weeks to a primary avian coccidia infection after oral vaccination (Martins *et al*., 2022). B cells haven't been very important in the development of coccidian resistance (Rasheed, 2016).

Vaccine development in its original form, as pioneered by Jenner and Pasteur, involved introducing an attenuated or inactivated antigen to mimic infection and induce immunity without causing disease (Viljoen *et al*., 2021). Based on components used in the synthesis of vaccines, specific characteristics, and the spectrum covered by the organism, vaccines are categorized into three generations (Ojha and Prajapati, 2022). The first-generation (conventional) vaccines, consist of whole microorganisms that have been non- attenuated or live-attenuated; second-generation (subunit) vaccines, primarily made of protein components like protein antigens or recombinant proteins; and third-generation vaccines, which consist of nucleic acid (DNA and mRNA) and recombinant vector vaccines, to elicit an immune response against a particular pathogen (Abdelaziz *et al*., 2024).

All vaccines now in use are based on live virulent and live attenuated formulations (Sharma *et al*., 2015), except for CoxAbic®, subunit vaccines developed via recombinant DNA technology (Gao *et al*., 2024). The production of live Eimeria vaccines includes non-attenuated and attenuated (Ahmad *et al*., 2016; Pastor-Fernández *et al*., 2020). Launched in the 1950s, CocciVac® (Alabama, USA) was the first coccidian vaccine. It used a sporulated oocyst of a live *E. tenella* wild-type strain (Zaheer *et al*., 2022). The live oocyst vaccines have a short shelf life, and their proper dosages are difficult to control. The live oocyst vaccines themselves can create a significant risk for coccidiosis outbreak (Nasri *et al*., 2022).

2.2.1. Attenuation methods of Eimeria vaccine production

Attenuated vaccines consist of Eimeria spp. isolates that have been altered in the laboratory to reduce their virulence and safer for use in chickens (Barbour *et al*., 2015). It is achieved through genetic manipulation, repeated passage, irradiation, chemicals, heat, and vaccines based on live strains that are relatively tolerant of ionophores (Falsafi *et al*., 2023). Vaccination with attenuated parasites has been proven more effective (Shakoor *et al*., 2021) than genetically engineered vaccines. Attenuating a parasite via serial passages in vitro or in vivo is laborious and not always successful. A well-known alternative method is chemical and ionizing radiation (mostly gamma rays) (Finkensieper *et al.*, 2023).

Formalin attenuation methods: Chemical attenuation involves Eimeria spp. strains altered in the laboratory to reduce their virulence, thereby enhancing protective immunity against antigens. Formalin is a substance used for this purpose. It interacts with amino and amide groups in proteins, as well as with amino groups bound to non-aqueous substances, which include the vital components of purine and pyrimidine nucleic acids (Anggraini *et al*., 2021). This reaction dehydrates the cells of the organism, leading to desiccation and structural stability. These organisms lower their pathogenicity without causing disease, and are highly effective in antibody formation (Setyowati *et al*., 2019).

Gamma ray attenuation methods: Radiation attenuation maintains the pathogens' ability to multiply and sustain metabolic activity while preserving their protein structures and preventing disease (Falsafi *et al*., 2023). Ionizing radiation primarily targets nucleic acids in living organisms, leaving most protein structures intact (Finkensieper *et al*., 2023). Despite these promising developments, no irradiated vaccines have yet been commercialized for use in poultry farming (Abdelaziz *et al*., 2024).

2.2.2. Efficacy studies of formalin and gamma ray attenuated vaccines

The criteria used to determine the effects of a challenge infection in immunized chickens are similar to those used to evaluate drug efficacy (Soutter *et al*., 2020). It is possible to include performance parameters of the chickens, such as greater survival rates, reduced oocyst shedding in feces, increased feed conversion ratios, reductions in the severity of intestinal lesions, and improved body weight gains relative to the control chickens *(Lee et al*., 2022). The vaccine trials in chickens still lack specific immunological assays for accurately predicting the protective efficacy of vaccines (Nasri *et al*., 2022).

Formalin potentials in the pathogenic attenuation of *E. tenella* with 0%, 0.15%, 0.3%, 0.6%, and 1.2% decreased oocyst production (Anggraini *et al*., 2021), reduced cecum lesions score without challenge infection (Setyowati *et al*., 2019), and reduced lesions score after challenge infection in broiler chickens (Armiani *et al*., 2019). Among the tested concentrations, 1.2% formalin was found to be the most suitable for achieving effective attenuation of *E. tenella*. The oocysts of *E. maxima*, *E. acervulina*, and *E. tenella* were exposed to gamma radiation ranging from 0-500 Gray. Immunization of birds with oocysts receiving 150 Gray was more effective in protecting from the negative effects of challenge infection as measured by feed conversion rate, changes in weight gain, lesion scores, and measurement of body composition (Fetterer *et al*., 2014). Thabet *et al*. (2019) reported that chickens immunized with *E. tenella* oocysts exposed to low-energy electron irradiation at doses between 0.1 kGy and 0.5 kGy exhibited reduced oocyst shedding and lesion scores, along with increased weight gain following challenge infection, compared to the positive control.

Vaccine challenge studies in laboratory trials: Animal welfare must be the priority in all research involving live animals and must be carefully planned. Replace, reduce, and refine animal utilization are the 3Rs that need to be considered (Tannenbaum and Bennett, 2015; Zaheer *et al*., 2022). Eimeria has inefficient in vitro life cycle completion capabilities. The choice of chicken breed or line in an Eimeria vaccine challenge trial is influenced by factors like relevance, experimental design, monitoring outcomes, and genetic background (Soutter *et al*., 2020).

While rodent models can be used to study responses to coccidial infection, they are unlikely to completely replace the need for studies in chickens because to the fact that rodent immune systems differ greatly from those of chickens (Nochi *et al*., 2018), and rabbit used for challenge trial by Juárez-Estrada *et al*. (2021). Moreover, a study in mice demonstrated that immunization with gamma ray irradiated *E. papillata* sporulated oocysts resulted in a reduction in oocyst output following experimental infection (Saleh *et al*., 2011). The degree of the disease and the effectiveness of immunization are affected by differences in fecundity, pathogenicity, and immunogenicity across Eimeria species. These variations determine the size of the challenge dose and the methodology of the study (Soutter *et al*., 2020).

Challenge doses should be experimentally established based on the species of Eimeria and the breed of chicken. They should also be customized to the study design so that the desired outcome of vaccination can be measured reliably and at an appropriate time point post-infection (Nolan *et al*., 2015). Oral gavage of oocysts is typically used to administer the challenge dosage, which can be given as a single dose or in multiple doses to attempt to replicate a natural infection (Soutter *et al*., 2020). Injectable, topical, and ocular delivery methods are available for live vaccine (Jenkins *et al*., 2013). When chicks are vaccinated with gel rather than spray, the amount of oocysts shed is around seven times higher (Elnaggar *et al*., 2022).

2.2.3. Advantages and disadvantages of formalin and γ- ray attenuated anticoccidial vaccines

Vaccination remains one of the most effective and successful methods against coccidiosis because vaccines provide a high level of protection against coccidiosis and replace the wild populations of Eimeria, which reduces the drug resistance problem in chickens (Khater *et al*., 2020). When chickens are exposed to low numbers of Eimeria parasites, birds develop immunity after two to three repeated infections, which demonstrates that vaccination is a reliable method (Shah *et al*., 2014). Attenuated strains can effectively stimulate the host immune system, promoting the production of antibodies and immune cells without causing significant intestinal damage (Zhao *et al.*, 2024).

However, certain limitations remain with current attenuation methods. A standard set of guidelines based on the modern vaccines in poultry Eimeria is lacking, but some countries for avian vaccines of bacterial or viral origin can have (Chapman *et al*., 2005). This causes differences in the study designs, such as the difference in vaccine formulation, inadequate formulation, vaccine schedule, challenge dose, and type or breed of chickens used, resulting in the production of no potent vaccine (Sharif and Ahmad, 2018; Soutter *et al*., 2020).

Attenuation by formalin has an impact on the antigenic structure of the pathogen and a more significant variability in reproducibility and reduced viability, in addition to the potential contamination with chemical residues (Djemai *et al*., 2023; Abdelaziz *et al*., 2024). Gamma ray attenuation requires effective shielding to mitigate health risks and faces certain technical limitations. As an alternative, high-energy electron beam irradiation has been proposed for pathogen attenuation; however, this method also demands substantial and costly shielding (Thabet *et al*., 2019).

3. MATERIALS AND METHODS

3.1. Study Area

The experimental study was conducted from November 2024 to May 2025 at the College of Veterinary Medicine and Agriculture (CVMA) in Bishoftu town. Bishoftu is located in the East Shewa zone of the Oromia regional state of Ethiopia, approximately 47 km southeast of Addis Ababa (**Figure 2**). For the experimental study, mixed species of coccidia oocysts were collected from commercial poultry farms in Bishoftu. The mean annual minimum and maximum temperature in Bishoftu are 14 and 26 °C, respectively, and the average relative humidity is 61.3% (Kassaw *et al*., 2022).

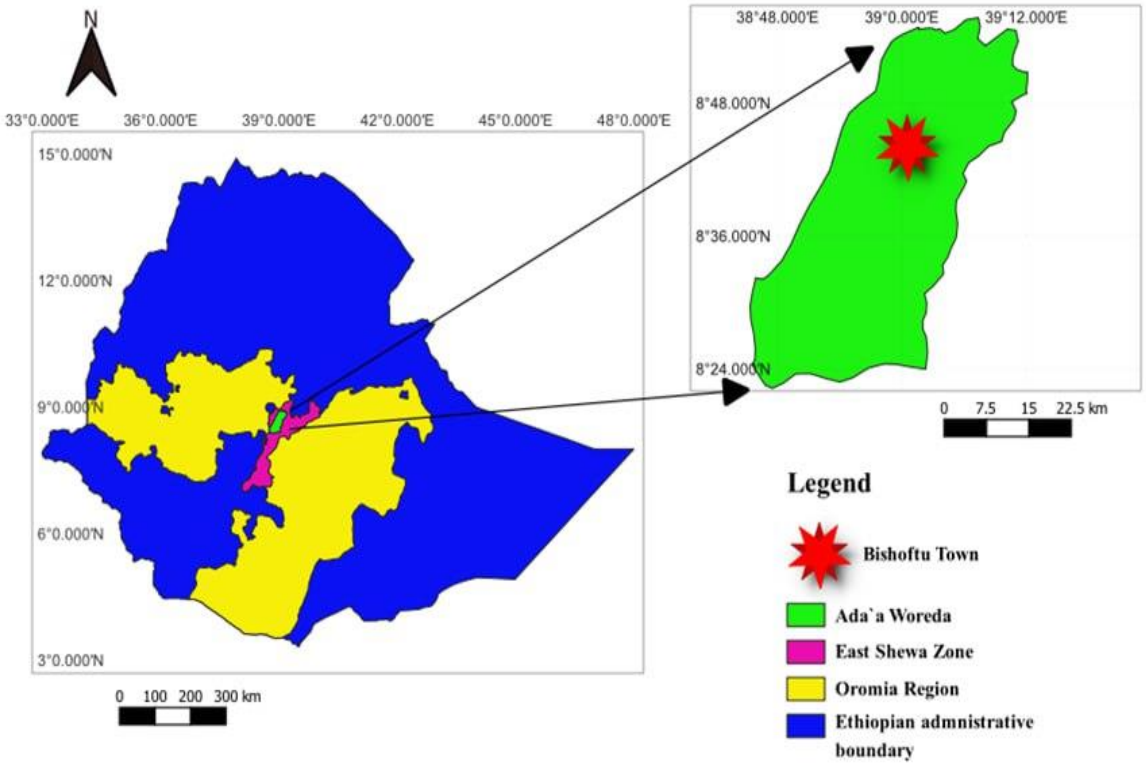

Figure 2: Map showing the study area.

3.2. Sample Collection and Sporulation of Oocysts

Coccidia infection suspected farms were identified based on clinical signs reported by commercial farms during farm visits. Upon permission from suspected farms, pooled fecal samples were collected from the litter and transported to the Parasitology Laboratory of the CVMA. The presence of Eimeria oocysts (without species identification) was determined using the fecal floatation technique. Eimeria oocysts were purified from the positive faecal samples following a modified protocol described by Eckert *et al*. (1995). Briefly, faecal samples were diluted with two volumes of tap water, homogenized and filtered through a 250-µm pore size sieve over double layer of gauze. The filtrate was left to sediment overnight, after which the supernatant was discarded. The remaining sediment was resuspended in saturated saline and centrifuged at 1300×g for 10 min. Suspended oocysts were collected and washed three times with tap water by centrifugation at 1300×g for 10 min. Finally, purified oocysts were poured into petri-dishes and mixed with 2.5% potassium dichromate ($K_2Cr_2O_7$) solution. The mixture was kept at room temperature for ten days with frequent shaking and aeration to allow maximum sporulation. Then oocysts were counted by Neubauer haemocytometer to determine sporulation percentage. On the 10^{th} day of incubation, above 85% of sporulation was reached. The suspension was tightly stoppered and stored at +4 °C until it was used for propagation (Kalkal *et al*., 2021).

3.3. Propagation and Purification of Oocysts

To obtain sufficient oocysts for vaccine preparation and experimental infection, propagation of Eimeria oocysts isolated from farm fecal samples were done in 15 Cobb500 broiler chickens drenched with $1.5x10^5$ oocysts at the age of three weeks. The chickens were fed coccidiostat-free feed and provided with potable water ad libitum (Elhassan *et al*., 2020). From the 7^{th} to 10^{th} day post-infection, as much volume of fecal droppings as possible was collected and processed as described before for oocysts sporulation and purification. The collected oocysts were kept at 4 °C until they were used for vaccine preparation (Ryley *et al*., 1976).

3.4. Formalin Attenuation of Sporulated Oocysts

Sporulated oocysts were cleaned with distilled water three times to remove $K_2Cr_2O_7$ solution. Finally, the oocyst pellet was soaked in 200 µL of 1.2% formalin (37% formaldehyde) for 96 h. Then, the oocysts were washed five times with distilled water by centrifugation at 1500×g for 10 min to clean them from formalin, the supernatant discarded, re-suspended in distilled water, and stored at 4 °C until used (Armiani *et al*., 2019; Setyowati *et al.*, 2019; Anggraini *et al*., 2021).

3.5. Gamma Ray Attenuation of Sporulated Oocysts

Sporulated oocysts were washed three times (1500×g, 5 min) with sterile distilled water to remove the 2.5% $K_2Cr_2O_7$, and re-suspended in 5 ml sterile distilled water. Finally, the oocyst suspension was adjusted to approximately 2×10^6 oocysts/ml. The adjusted suspension was placed in a universal bottle. The γ-irradiation process was conducted at the National Institute for Control and Eradication of Tsetse and Trypanosome (Addis Ababa, Ethiopia) in the Gammacell220® (MDS Nordion, Canada) using cobalt 60 as a source of radiation to a dose rate of 150 Gray. The temperature range of the gamma chamber was maintained at 37-40 °C. After completion of the irradiation process, each tube was carefully taken out of the gamma chamber and immediately stored at 4 °C until used. A non-irradiated oocyst was used as a control. The attenuation capacity of radiation dose was evaluated by acute safety test (Dieu and Luc, 2001; Fetterer *et al*., 2014).

3.6. Acute Vaccine Safety Test

To ensure the live attenuated vaccine is safe, three groups of five chickens were used. Chicken in two groups are drenched with $1x10^5$ attenuated sporulated oocysts per chicken in 1 ml from each vaccine type. The third group served as unvaccinated control and received distilled water in place of the vaccine. Following vaccination, general behavior, signs of illness, feed intake, and fecal consistency were monitored daily for ten days. Absence of serious signs of illness on live chicken and severe intestinal gross lesions at postmortem indicates that the vaccine is safe.

3.7. Experimental Design

The study design involves a randomized controlled trial to assess the efficacy of the attenuated coccidial vaccine against challenge infection in experimental chickens. Each chicken was marked with a unique identification on its head. A lottery system was used to randomly allocate chickens to treatment and control groups. The protective efficacy of the two attenuated oocysts preparations was evaluated against the non-vaccinated control groups.

3.8. Experimental Chicken and Vaccination Schedule

One hundred fifty (150) day-old Cobb500 breed of broiler chicks were purchased from Alema, a private commercial broiler farm, Bishoftu, Ethiopia. The unsexed day-old chickens with an average weight of 49.25 g were blocked for their body weight and randomly allocated into ten groups of 15 birds (five in three replicates). Group FR_{500}-NCH and FR_{1000}-NCH received 500 and 1000 formalin attenuated oocysts orally in 0.1 ml respectively; group GR_{500}-NCH and GR_{1000}-NCH received 500 and 1000 γ-ray attenuated oocysts respectively but all were not challenged afterwards. On the other hand, group FR_{500}-CH and FR_{1000}-CH received formalin attenuated oocysts whereas groups GR_{500}-CH and GR_{1000}-CH received γ-ray attenuated oocysts with similar doses as above followed by a challenge infection by field strain of the same parasite isolate at a dose of $1x10^5$ sporulated oocysts in 1 ml of distilled water. In addition, group NV-PC not received either of the vaccines followed by a challenge infection (positive control), while group NV-NC received neither the vaccine nor the challenge infection and hence served as a negative control to check against any contamination (**Table 2**). Oral vaccination was given at days 4 and day 11 of age, while challenge infections were done on the 21st day of age.

Table 2: Experimental chicken grouping and activity schedule

D1: Grouping	D1-3	D4, D11 Day 0 & D7 PIV	D4, D7, D11, D14	D21 D0-PCH	D21 D0-PCH	D24, D28, D31	D31 D10-PCH
FR_{500}-NCH	Acclimatization, Fecal exam, Body weight measurement	BW, Vac-FR500	BW, FE, CO, MR	BW, CO, FE, PM1	DW	BW, FE, CO, MR	PM2
GR_{500}-NCH		BW, Vac-GR500	BW, FE, CO, MR	BW, CO, FE, PM1	DW	BW, FE, CO, MR	PM2
FR_{1000}-NCH		BW, Vac-FR1000	BW, FE, CO, MR	BW, CO, FE, PM1	DW	BW, FE, CO, MR	PM2
GR_{1000}-NCH		BW, Vac-GR1000	BW, FE, CO, MR	BW, CO, FE, PM1	DW	BW, FE, CO, MR	PM2
FR_{500}-CH		BW, Vac-FR500	BW, FE, CO, MR	BW, CO, FE, PM1	CH 10^5	BW, FE, CO, MR	PM2
GR_{500}-CH		BW, Vac-GR500	BW, FE, CO, MR	BW, CO, FE, PM1	CH 10^5	BW, FE, CO, MR	PM2
FR_{1000}-CH		BW, Vac-FR1000	BW, FE, CO, MR	BW, CO, FE, PM1	CH 10^5	BW, FE, CO, MR	PM2
GR_{1000}-CH		BW, Vac-GR1000	BW, FE, CO, MR	BW, CO, FE, PM1	CH 10^5	BW, FE, CO, MR	PM2
NV-PC		BW, DW	BW, FE, CO, MR	BW, CO, FE, PM1	CH 10^5	BW, FE, CO, MR	PM2
NV-NC		BW, DW	BW, FE, CO, MR	BW, CO, FE, PM1	DW	BW, FE, CO, MR	PM2

Key: *BW: body weight, CH: challenge, CO: clinical observation, DW: distilled water, FE: Fecal examination, FR: formalin, GR: Gamma ray, MR: mortality record, NCH: Non-challenge, NV-NC: non-vaccinated non-infected control, NV-PC: non-vaccinated infected control, PM: postmortem, Vac-FR: formalin attenuated vaccine, Vac-GR: γ-ray attenuated vaccine*

3.9. Experimental Chicken Management

The chickens were kept in a cleaned and disinfected animal house located in the CVMA. All the chickens were kept on coccidiosatt free broiler starter ration for ten days of age and then provided with a grower feed from 11-30 days of age and finisher feed for the remaining days. Water was provided ad libitum. All the experimental chickens were vaccinated against Newcastle disease (Lasota) (tenth and 20^{th} day old) under the recommended vaccination program. The chickens were reared in a deep litter system. In

addition to putting physical barriers, a foot bath was placed at the entrance of the experimental unit to ensure biosecurity. Personal protective clothes, including face masks, were used by all people in contact with the chicken. To avoid the spread of the vaccine strains to non-vaccinated birds, vaccinated groups were isolated from the other groups by empty pens and walls of a plastic sheet. Separate working clothes and tools were used for each area. All pens were heated using a 200W electric bulb to maintain the temperature within 25-30 ^{o}C depending on the age of chicken (Sharaban *et al*., 2021).

3.10. Evaluation of Attenuated Vaccine Efficacy

3.10.1. Fecal oocyst count

Daily monitoring of all experimental chickens was conducted daily for the presence of any clinical signs and mortality. Three fresh fecal samples were collected from the floor of each group, processed by the simple floatation technique, and oocyst per gram (OPG) was estimated using the McMaster egg counting slide according to Haug *et al*. (2006). Then the relative oocysts count was compared between vaccinated and non-vaccinated groups as follows:

$$\text{Relative oocyst production (ROP)} = \frac{\text{OPG of vaccinated group}}{\text{OPG of NV-PC group}} \times 100$$

Where NV-PC= non-vaccinated positive control (challenged)

3.10.2. Body weight gain

Body weight measurements were done as per the schedule given in table 2. A sensitive balance was used to weigh individual chickens to the nearest gram in the morning before feeding and the values were compared among vaccinated and unvaccinated control groups as follows:

$$\text{Relative rate of body weight gain (RRBWG)} = \frac{\text{\% change in BW of vaccinated group}}{\text{\% change in BW of NV-NC group}}$$

$$\text{Growth and survival ratio (GSR)} = \frac{\text{Final body weight (D10-PCH) + BW of dead birds}}{\text{Initial body weight (D0-PCH)}}$$

Where PCH=post challenge, NV-NC= non-vaccinated, negative control

3.10.3. Determination of chicken survival rate

Chicken mortality was recorded as previously described (Ojimelukwe *et al*., 2018) starting from the day of the first vaccination up until the end of the experiment. Data was used to calculate chicken survival rate in the presence or absence of coccidal vaccination as follows:

$$\text{Survival rate} = \frac{\text{Number survived at D10-PCH}}{\text{Number at D0-PCH}} \times 100$$ where, PCH= post challenge

3.10.4. Gross lesion score

Cervical dislocation technique was employed to humanely euthanize experimental chickens. After opening the abdominal area, the intestines were recovered and examined for gross lesions. Since major gross lesions were observed only on the caeca, lesion scores were done on caecal lesions on the day of challenge to see the effect of vaccination and on D10 post challenge to see the role of prior vaccination in reducing intestinal lesions (Johnson and Reid, 1970)). During both postmortem periods, three randomly selected chickens from each group were sacrificed. A score of '0' was given to normal cecum; '1' when small scattered petechiae were observed; '2' when numerous petechiae were detected; '3' when extensive hemorrhage was apparent, and '4' when extensive hemorrhage associated with dark and thickened intestinal mucosa was present.

3.10.5. Final estimation of Vaccine efficacy

Lan *et al*. (2017) has formulated estimation of vaccine efficacy based on four parameter or indices: Anticoccidial Index (ACI), Relative Oocyst Production (ROP), Percent Optimum Anticoccidial Activity (POAA) and Reduction of Lesion Score (RLS). In this study, these four indices were calculated from oocyst values (OPG), body weights (BW), chicken survival rates (SR), growth and survival rate (GSR) and lesion scores (LS).

- The ACI was calculated according to the following formula (Mi *et al*., 2024):
  ACI= (Relative rate of weight gain+ survival rate) − (lesion score + oocyst value)
  ACI >180 means adequate protection; 160 < ACI < 179 means moderate protection; and ACI< 160 shows no protective effect

- **ROP:** the relative oocysts population was compared between vaccinated and non-vaccinated groups as previously described.

  ROP < 15% shows protective efficacy and ROP ≥ 15% indicates the vaccine was not protective effective.

- **POAA:** Percent optimum anticoccidial activity was calculated as follows:

$$\text{POAA} = \frac{(\text{GSR in the vaccinated group} - \text{GSR in the NV-PC group}) \times 100}{(\text{GSR in the NV-NC group} - \text{GSR in the NV-PC group})}$$

  POAA value ≥ 50% shows vaccine has protective efficacy

- **RLS:** Reduction in lesion scores was estimated as follows

$$\text{RLS} = \frac{\text{Average LS of NV-PC} - \text{Average LS of vaccinated group}}{\text{Average LS of NV-PC}} \times 100$$

  If the RLS value is ≥ 50%, it shows protective efficacy.

Overall judgment: if all or three of the values fall in the protective category, the vaccine is considered as adequately protective. If two of the four indices show protection, it implies that the vaccine is moderately effective. Any observation below this, testifies that the vaccine under consideration is slightly protective or not protective at all (Lan *et al*., 2017).

3.11. Statistical Analysis

The collected data was stored in a spreadsheet program (excel) and analysed using by the software R 4.3.1 statistical software. The body weights and oocyst per gram of feaces were analysed by one-way ANOVA and a post hoc analysis using Tukey's multiple comparison test to identify statistically significant variations. Gross intestinal lesion was analyzed by using t-test pairwise comparison. Chicken survival rate was estimated in terms of percentage. Finally, vaccine efficacy assessment involves determining different indices based on the values of body weight, lesion score, survival rate, and oocyst count. This part used descriptive statistical analysis. Data was expressed as the mean ± standard deviations, considered significant at < 5% ($p < 0.05$).

3.12. Ethical Clearance

All experimental procedures and chicken management were compliant with internationally recognized guidelines regarding research animal handling and use. This research project is part of the thematic research project “Integrated management of Poultry coccidiosis (IMPCOC)” which has an ethical clearance certificate (Ref. No.VM/ERC/03/31/16/2024) obtained (**Annex 6**) from the animal research ethics committee of the College of Veterinary Medicine and Agriculture (Addis Ababa University).

3.13. Limitations of the Study

Due to financial, time and ethical constraints, different intensity of exposure of formalin and gamma rays was not tried in the production of attenuated vaccines. Moreover, although gross intestinal lesions have commonly shown caecal coccidiosis, techniques such as molecular methods were not available which may have supported in the identification of the parasite at species level.

4. RESULTS

4.1. Vaccine Acute Safety Test

Those chickens in the non-vaccinated control group exhibited a healthy appearance with good appetite throughout the one-week observation period. Drenched chicken, in the first four days following vaccine dosing, no animal has shown any sign of abnormality. In contrast, vaccinated chickens showed mild clinical signs such as blood tinged watery feaces, depression, ruffled feathers, and huddling behavior thereafter. In both vaccinated groups (formalin treated and γ irradiated), OPG was highest on day seven (**Table 3**), but higher in those that received γ-ray attenuated oocyst. Gross lesion examination at postmortem on 10^{th} day post-infection has shown mild petechial haemorrhage in the caeca of both vaccinated groups. However, no animal died during the observation period and body weights were not different from the control group.

Table 3: Average oocyst shedding in chickens dosed with formalin and γ-ray attenuated Eimeria oocysts in the first seven days post-infection.

Group	**5^{th} day**	**6^{th} day**	**7^{th} day**
Formalin	-	$10x10^4$	$18.22x10^4$
Gamma ray	$31.5x10^2$	$14.066x10^4$	$26.88x10^4$

4.2. Responses of Experimental Chickens to Vaccination

4.2.1. Clinical examination

All vaccinated chicks were monitored from day 1 to day 4 post-initial vaccination (PIV) and showed no visible clinical signs. However, the following morning, some of them showed mild blood tinged watery feaces, and this was more common in the GR_{1000} groups, but the sign disappeared by the next day. After booster vaccination (7 days from initial vaccination), none of the vaccinated chickens displayed visible clinical signs. On the other hand, all chicken in the NC-NC group remained normal throughout the experimental period. After challenge infection, challenged chickens showed mild clinical

signs of coccidiosis, which appeared at the 5th day of challenge. A few chickens in vaccinated challenged groups showed watery feces that were tinged with blood and signs of depression, with these signs being more frequent in the FR_{500} group. Clinical signs were much severe in the NV-PC group, with obvious bloody diarrhea, huddling together, ruffled feathers, soiled vent area and some mortality that continued up to day nine post-challenge (PCH).

4.2.2. Analysis of fecal oocyst output

No coccidia oocyst was detected in the NV-NC group throughout the experimental period (**Table 4**). Vaccinated but not challenged groups (FR_{500}-NCH, FR_{1000}-NCH, GR_{500}-NCH and GR_{1000}-NCH) have started shedding oocysts 7 days post initial vaccination (7D-PIV, 11d old) and continued to do so with decreasing oocyst output until the end of the experiment. In a similar manner, challenge groups (FR_{500}-CH, FR_{1000}-CH, GR_{500}-CH and GR_{1000}-CH) have started oocyst production in the same pattern as in non-challenge vaccinated groups until challenge dose is given (0D-PCH, 21d old) with local isolates of sporulated oocysts.

Following challenge infection of vaccinated groups and the non-vaccinated positive control group (NV-PC), varying degrees of oocyst production was observed throughout the remaining part of the experimental period. Accordingly, at both day 7 and day 10 PCH, the NV-PC group exhibited significantly higher OPG compared to the vaccinated-challenged groups (FR_{500}-CH, FR_{1000}-CH, GR_{500}-CH and GR_{1000}-CH groups ($p<0.05$). Among vaccinated and then challenged groups, GR_{1000}-CH group followed by GR_{500}-CH had significantly reduced oocyst count compared to others ($p<0.05$).

Table 4: The mean number of oocysts output across experimental groups at various time intervals

Groups	7D-PIV (V2 dose)	10D-PIV	0D-PCH (challenge)	3D-PCH	7D-PCH	10D-PCH
FR_{500}-NCH	25966.67±1050.39^{b}	7166.67±351.18^{c}	1183.33±28.87^{d}	3200±50^{f}	633.33±144.33^{a}	350±50^{a}
GR_{500}-NCH	49933.33±1401.19^{c}	966.67±76.37^{a}	650±50^{b}	733.33±76.37^{c}	666.67±76.37^{a}	333.33±76.37^{a}
FR_{1000}-NCH	99466.67±702.37^{d}	32133.33±665.8^{d}	4966.67±76.37^{e}	4316.67±76.37^{g}	900±132.28^{a}	533.33±125.83^{a}
GR_{1000}-NCH	150366.7±757.18^{e}	4283.33±325.32^{b}	2800±132.29^{c}	2133.33±144.33^{e}	1066.67±28.86^{a}	500±50^{a}
FR_{500}-CH	25000±1228.82^{b}	8266.67±450.92^{c}	1316.67±76.37^{d}	916.67±125.83^{bc}	82666.67±2939.95^{e}	12200± 888.81^{d}
GR_{500}-CH	52733.33±1537.31^{c}	650±50^{a}	850±50^{b}	150±50^{a}	36566.67±1858.31^{c}	4200±264.57^{b}
FR_{1000}-CH	98266.67±1201.38^{d}	33066.67± 686.6^{d}	4533.33±404.14^{e}	1933.33±104.08^{de}	48666.67±3302.02^{d}	7116.67±425.24^{c}
GR_{1000}-CH	149566.7±850.49^{e}	3083.33±152.75b	3133.3±104.08^{c}	866.67±104.08^{bc}	10400±1135.78^{b}	1283.33±125.83^{a}
NV-PC	0^{a}	0^{a}	0^{a}	0^{a}	298800±5367.49^{f}	45200±1670.32^{e}
NV-NC^{a}	0	0	0	0	0	0

Data were presented as mean ±SD. Values within the same columns with different letters differ significantly at $P \leq 0.05$.

Key: *CH: Challenge, FR: Formalin, GR: Gamma ray, NCH: Non-challenge, NV-NC: Non-vaccinated non-infected control, NV-PC: Non-vaccinated infected control, PCH: Post-challenge, PIV: Post initial vaccination, V2: 2nd vaccination*

4.2.3. Body weight measurement

Body weight (BW) was measured at specific time points; between the day of first vaccination and day of challenge dose and between the day of challenge dose and end of the experiment (**Table 5**). The average BWs of all experimental groups at the time of initial vaccination (0D-PIV) when the chicken were 4 days old were statistically similar. Likewise, mean BWs among vaccinated groups were not statistically different at the time of challenge dose administration (0D-PCH, chicken age 21 days). On the other hand, BWs of the non-vaccinated groups (NV-PC and NV-NC) were significantly higher than those of the vaccinated groups ($P<0.05$) on the day of the challenge infection.

Following challenge infection, however, mean BW varied among different groups both 7 days and 10 days post challenge. Accordingly, the NV-NC had significantly higher body weight compared to all other groups ($P<0.05$). The next best mean BW among challenged groups was registered for those chickens that received 1000 attenuated oocysts (FR_{1000}-CH and GR_{1000}-CH) while the lowest BW was registered for the non-vaccinated challenged, positive control group (NV-PC).

Table 5: Average body weight in different groups according to schedule

Groups	0D-PIV (V1 dose)	3D-PIV	7D-PIV (V2 dose)	10D-PIV	0D-PCH (Challenge)	3D-PCH	7D-PCH	10D-PCH
FR_{500}-NCH	69.86±4	104.4±4.8	173.67±10	215.67±7.33^{b}	374.56±30.68^{b}	531±27.62^{b}	630.5±14.73^{b}	727± 6.38^{b}
GR_{500}-NCH	71.33±3.0	104.27±3.3	163.8±12.5	207.01±10.83^{b}	360.05±11.52^{b}	496.44±15.07^{bc}	598.05±10.05^{bcd}	700.53±10.44^{bcd}
FR_{1000}-NCH	68.67±5.9	99.73±3.4	159.98±4	213.69±5.8^{b}	344.35±4.50^{b}	487.33±20.25^{bc}	605.94±10.51^{bc}	704.67±10.21^{bc}
GR_{1000}-NCH	70.27±2.9	101.27±5.4	158.6±3.6	203.81±3.10^{b}	371.53±6.76^{b}	512.33±15.04^{bc}	615.56±12.16^{bc}	711.89±12.38^{bc}
FR_{500}-CH	70.33±2.4	99.53±3.7	170.4±4.7	209.58±7.50^{b}	356.21±6.36^{b}	458.75±21.24^{bc}	529.17±21.00^{g}	585.03±26.18^{fg}
GR_{500}-CH	69.87±4.4	99.13±7.8	160.8±9.8	210.75±7.55^{b}	348.28±12.03^{b}	459.42±9.41^{c}	532.67±8.01^{fg}	606.08±17.04^{fg}
FR_{1000}-CH	69±2.6	103.8±7.7	159.4±3	208.2±16.31^{b}	372.88±16.63^{b}	478.36±18.31^{bc}	572.14±16.91^{ef}	616.17±17.32^{efg}
GR_{1000}-CH	72.2±5.8	99.07±3.1	158.53±8.7	207.42±8.93^{b}	348.42±17.82^{b}	486.51±10.89^{bc}	587.13±4.34^{de}	675.22±14.00^{cde}
NVPC	67.27±3	106.73±4.2	178.67±13.5	249.2±6.96^{a}	401.47±6.20^{a}	457.87±24.00^{c}	473.67±16.58^{h}	579.78±16.21^{g}
NVNC	66.13±1.8	111.13±7.9	177.2±11.5	252.4±9.90^{a}	405.33±18.10^{a}	611.33±19.18^{a}	685.17±19.26^{a}	799.5±10.98^{a}

Data were presented as mean ±SD. Values within the same columns with different letters differ significantly at $P \leq 0.05$.

Key: *CH: challenge, FR: Formalin, GR: Gamma ray, NCH: Non-challenge, NV-NC: Non-vaccinated non-infected control, NV-PC: Non-vaccinated infected control, PCH: Post-challenge, PIV: Post of initial vaccination, V1: 1st vaccination, V2: 2nd vaccination*

4.2.4. Determination of survival rate

Survival rate is summarized in **Table 6**. No mortality was observed in the NVNC group throughout the study period. All vaccinated groups achieved a 100% survival rate. In contrast, the NVPC showed 80% survival rate after the challenge infection.

Table 6: Survival rate at the end of the experiment in relation to the number of chickens on the day of challenge infection

Groups	0D-PIV	0D-PCH	10D-PCH	% survived
FR_{500}-CH	15	14	14	100
GR_{500}-CH	15	14	14	100
FR_{1000}-CH	15	14	14	100
GR_{1000}-CH	15	14	14	100
NV-PC	15	15	12	80
NV-NC	15	15	15	100

Key: *FR: Formalin, GR: Gamma ray, NV-NC: Non-vaccinated negative control, NV-PC: Non-vaccinated positive control, PCH: Post-challenge, PIV: Post-initial vaccination.*

4.2.5. Evaluation of gross intestinal lesion

Chicken were randomly euthanized on the day of first vaccination (0D-PIV, 4 days old) to check that all chicken were normal at the start of the experiment, at the age of 21 days just before challenge infection (0D-PCH) to see changes due to vaccination dose, and 10 days after (10D-PCH) to asses protective role of vaccination against challenge infection. Observations for gross pathological changes showed no intestinal lesions in group NV-NC at all examination points. In contrast, variable degrees of lesions were detected in the NV-PC and all vaccinated groups. Although slight changes were observed in different parts of the intestines of some chicken, marked changes were observed in the cecal region with higher scores in NV-PC group. Overall, the mean lesion score was increased in post-challenge than post-vaccination in both vaccinated and non-vaccinated groups, with the difference being statistically significant (paired $t=-3.3602$, $P < 0.05$) (**Figure 3**).

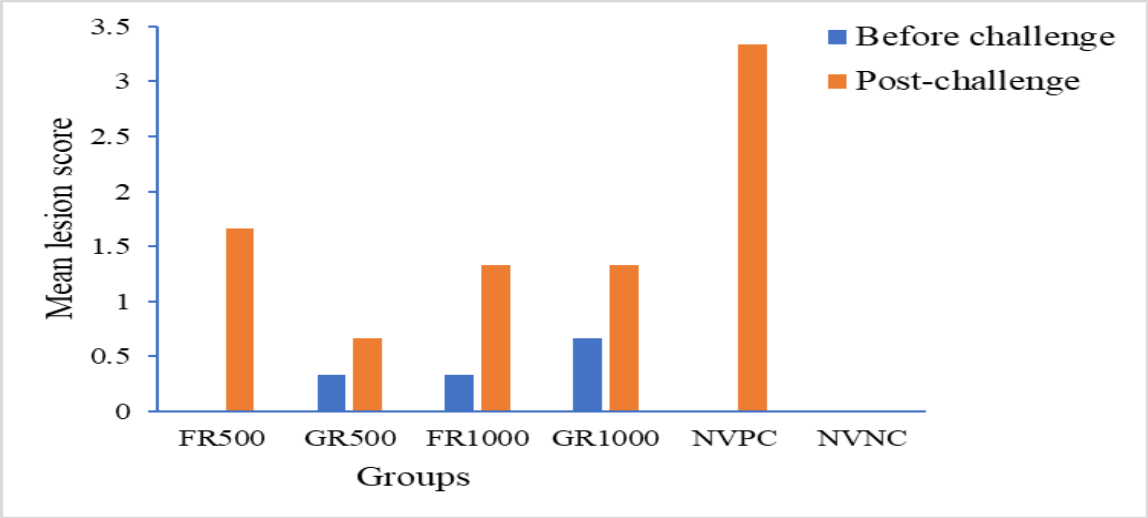

Figure 3: Mean lesion score before and post-challenge

Key: *FR: Formalin, GR: Gamma ray, NV-NC: Non-vaccinated negative control, NV-PC: Non-vaccinated positive control. The data represent mean.*

4.2.6. Efficacy of formalin and gamma ray attenuated oocyst vaccine

The four indices were estimated to determine anticoccidial vaccine efficacy in the vaccinated challenged groups. The findings show that (**Table 7**), both doses (500 and 1000/chicken) of the formalin attenuated sporulated oocysts fail to protect against challenge infection except the fact that they moderately reduced the relative lesion score (RLS). On the other hand, the γ-irradiated oocysts have unequivocally demonstrated excellent protective efficacy indices with all four parameters except for the GR_{500}-CH group which showed moderate performance with regards to ACI. Altogether, the analysis shows that γ-ray attenuated oocysts have promising performance as candidate vaccine.

Table 7: Coccidial vaccine efficacy index against challenge infection for formalin and γ-ray attenuated oocysts

Groups	Average BW on day of challenge (g)	Average BW on 10-day of PCH (g)	BW gain mean ±SD	GSR	%RRBWG	Survival rate (%)	Mean lesion score	OPG in feces 10-day PCH Mean ±SD	ACI		ROP (%)		POAA (%)		RLS (%)		Overall
									Value	Efficacy	Value	Efficacy	Value	Efficacy	Value	Efficacy	
FR_{500}	356.21 ±6.36	585.03 ±26.18	228.82 ±19.9^{cd}	1.64	66.06	100	1.67 ±1.52	12200 ±888.81	89.06	NP	26.99	NP	37.52	NP	50	P	NP
GR_{500}	348.28 ±12.03	606.08 ±17.04	257.8 ±9.97^{c}	1.74	76.12	100	0.67 ±1.52	4200 ±264.57	166.8	MP	9.29	P	56.03	P	80	P	P
FR_{1000}	372.88 ±16.63	616.17 ±17.32	243.28 ±22.72^{c}	1.65	67.09	100	1.3 ±1.15	7116.67 ±425.24	151.3	LP	15.74	NP	39.42	NP	60	P	NP
GR_{1000}	348.42 ±17.82	675.22 ±14.00	326.8 ±9.25^{b}	1.94	96.45	100	1.3 ±1.52	1283.33 ±125.83	193.6	P	2.84	P	93.47	P	60	P	P
NVPC	401.47 ±6.20	579.78 ±16.21	178.31 ±15.93^{d}	1.44	45.67	80	3.3 ±0.58	45200 ±1670.32	25.67		100		0		0		
NVNC	405.33 ±18.10	799.5 ±10.98	394.83 ±15.13^{a}	1.97	100	100	0	0	200		0		100		100		

Key: *ACI: Anticoccidial index, P: protective, BW: Body weight, FR: Formalin, GR: Gamma ray, GSR: Growth and survival rate, MP: Moderate protective, NP: Non-protective, NV-NC: Non-vaccinated non-infected control, NV-PC: Non-vaccinated infected control, OPG: Oocyst per gram, PCH: Post-challenge, POAA: Percent optimum anticoccidial activity, ROP: Relative oocyst production, RLS: Reduction of lesion score, RRBWG: Relative rate of body weight gain, SD: Standard deviation*

5. DISCUSSION

Eimeria species are obligate intracellular protozoan parasites that cause clinical avian coccidiosis. This condition affects the health, reduces production efficiency, and leads to major economic losses in the global poultry industry (Mi *et al.*, 2024). Vaccination is a proven and effective strategy against coccidiosis and reduces the risk of drug resistance. An ideal anticoccidial vaccine must be cost-effective, affordable and highly effective in protecting chickens from Eimeria infections (Tang *et al*., 2020; Zaheer *et al*., 2022). The current study was undertaken to evaluate the safety and efficacy of formalin and gamma ray attenuated coccidial vaccines produced from local isolates against challenge infection in broiler chicken.

The anticoccidial vaccine's safety must be shown by giving it to naïve birds at a dose at least ten times greater than the maximum amount found in a commercial batch, using the recommended method and age (Chapman *et al*., 2005). In this study acute vaccine safety test for formalin and gamma ray attenuated sporulated oocysts showed only mild clinical signs, low oocyst shedding, reduced intestinal lesion score and no mortality. This finding supports the report of Zhao *et al*., (2024) which documented that attenuated strains can cause mild infections during vaccination, stimulating the host's immune system to produce antibodies and immune cells, providing protection without causing severe intestinal damage.

Oral vaccination of broiler chickens with formalin or γ-ray attenuated vaccines with two different doses (500 and 1000 sporulated oocysts) at the age of 4 and 11 days followed by challenge infection by virulent sporulated oocyst on day 21 of age have induced variable degrees of protection. This align with the method and findings described by Arczewska-Włosek and ̄wiaętkiewicz (2014), where protective efficacy was reported against challenge infection. The protective efficacy of the attenuated vaccine was evaluated based on parameters such as oocyst output, body weight, survival rate, and macroscopic intestinal lesions in the study periods (Juárez-Estrada *et al*., 2021).

5.1. Performance of Attenuated Vaccines Compared to the Non-Vaccinated Group

This study has demonstrated that all vaccinated groups had lower oocysts counts, better body weight gains, improved survival rate and lower intestinal lesion scores following challenge infection compared to the non-vaccinated challenged group. This strongly suggests vaccinating chickens may provide some degree of protection against virulent infections. This finding agrees with several previous reports (Anggraini *et al*., 2021). Anticoccidial vaccines can mitigate the negative effects of coccidiosis, which often reduces the absorptive surface area of the intestine, impairs nutrient absorption and triggers inflammation (Lee *et al*., 2011).

The current findings agree with Thabet *et al*. (2019), who reported that chickens vaccinated with *E. tenella* oocysts that were attenuated through low-energy electron irradiation exhibited mild clinical signs without any life threatening effects. Gilbert *et al.* (1998) have shown that irradiated oocysts of laboratory and field isolates of *E. tenella* produced fewer oocysts. Fetterer *et al*. (2014) have also reported that chickens immunized with irradiated oocysts displayed lower lesion scores in all three regions of the intestine, but lesions were not eliminated following challenge infection. The result agrees with Jenkins *et al*. (1997), who report that immunized chickens with irradiated *E. maxima* oocysts reduced lesion scores over non-immunized chickens.

Vaccination has improved body weight gain because Eimeria infection compels chicken to redirect energy from growth to immune responses, thereby hindering weight gain (El-Ashram *et al*., 2019). In a similar manner, vaccinated groups showed few petechial haemorrhage, and slight thickening of caecal wall. This result agrees with Setyowati *et al*. (2019) who reported that formalin attenuation at 1.2% concentration could reduce ceacal lesions with slight discoloration of cecal wall contents. Armiani *et al*. (2019) also reported, attenuated *E. tenella* inoculated to CP 707 strain broiler chicken reduced pathological consequences of challenge infection leading to no visible lesion or wall thickening. Intestinal lesions were even lower in vaccinated chicken before the challenge compared to post-challenge, which indicates that the attenuated anticoccidial vaccine may have little or no effect on the lesion scores (Williams, 2002).

The present results indicated that the post-vaccination of oocyst output was low in γ-ray attenuated Eimeria oocysts vaccinated groups compared with control group. Findings align with those of Fetterer *et al*. (2014) and Nguyen *et al*. (2024). Another study has also documented that oocyst excretion following a pathogenic challenge was greatly reduced compared to non-immunized control birds (Jenkins *et al*., 2013). Passage of limited number of oocysts following vaccination means that the buildup of vaccinal oocysts in the litter of birds ensured active vaccine cycling which may contribute to enhanced immune responses.

5.2. Comparison of Formalin and γ-Radiation Attenuated Vaccines

The findings of this study have clearly shown that efficacies of attenuated vaccines were dose-dependent, with higher doses producing better protection. The result aligns with Rashid *et al*. (2012), who observed a significant reduction in the OPG count in broiler chickens vaccinated with a formalin-inactivated *E. tenella* vaccine compared to the control group. The chickens that received 1000 formalin attenuated Eimeria oocysts achieved a higher body weight gain compared with 500 oocysts dose and positive control group, which is in agreement with the report of Rashid *et al*. (2012), vaccination with 3% formalin inactivated oocysts improved body weight gain compared with infected control.

This study is able to demonstrate that oral vaccination of broiler chicks with 1000 γ-ray attenuated oocysts of Eimeria has greatly lowered number of oocysts shedding better than the $GR_{500,}$ $FR_{1000,}$ FR_{500} and NV-PC groups. This is in agreement with Fetterer *et al*. (2014), who observed that birds immunized with irradiated oocysts receiving 150 Gray had decreased litter oocyst counts by about 100-fold. Similarly, Thabet *et al*. (2019) have found that *E. tenella* oocysts exposed to low-energy electron irradiation at 0.1 kilogray and 0.5 kilogray had significantly lower oocyst outputs in all vaccinated groups compared to the positive control group.

The present study has also shown that chicken vaccinated with the 1000 γ-ray attenuated oocyst dose has resulted in highest body weight gain than in the other groups ($GR_{500,}$ $FR_{1000,}$ FR_{500} and NV-PC groups). Similar result were described by Fetterer *et al*. (2014),

who observed significantly greater weight gain of birds immunized with γ-irradiated oocysts compared to unimmunized birds after challenge infection. Jenkins *et al*. (1997) have also shown that chickens immunized with 17-kRad-irradiated *E. maxima* oocysts had higher average weights at challenge than those immunized with non-irradiated oocysts. In addition, Thabet *et al*. (2019) found that *E. tenella* oocysts exposed to low-energy electron irradiation at Paracox®-8 and 0.5 kilogray had higher weight gain in all vaccinated compared to the positive control group.

5.3. Overall Vaccine Effectiveness

Coccidial vaccine efficacy was evaluated using the four parameters described by Lan *et al.* (2017) and Mi *et al*. (2024), which include anticoccidial index (ACI), relative oocyst production (ROP), percent optimum anticoccidial activity (POAA) and reduction of lesion score (RLS). According to the results, the group received γ-ray attenuated Eimeria oocysts demonstrated protective efficacy, as all index values aligned with protective criteria except for the GR_{500}-CH group, which showed moderate performance with regards to ACI. The groups administered with both 500 and 1000 formalin-attenuated Eimeria oocysts showed limited or no protective efficacy, as more than two of the four indices fell within the non-protective range. Mi *et al*. (2024), by using the common *E. maxima* and *E. acervulina* Antigen Elongation Factor 2 as a vaccine candidate have reported anticoccidial index (ACI) scores of 166.35 and 185.08, showing moderate-to-excellent protective efficacy.

Although Anggraini *et al*. (2021) suggested that formalin soaking with a concentration of 1.2% was the most optimal concentration to attenuate *E. tenella*, this was not the case in the current study. The different could be due to variations in parasite virulence or management of chickens. Awade *et al*. (2019) has also demonstrated that immunization of rabbits by UV irradiated Eimeria oocysts showed low number of oocysts in feces before and after challenge compared to groups that received formalin attenuated oocysts, freezing-thawed oocysts or non-vaccinated challenged groups. The report added that the same group showed high level of IgG antibody. This strongly supports the current finding that formalin attenuation may provide lower protection.

6. CONCLUSION AND RECOMMENDATIONS

The objective of this study was to assess the protective efficacies of local isolates of sporulated Eimeria oocysts attenuated by either formalin or γ-radiation as potential vaccine candidates against poultry coccidiosis. it was observed that although both products had improved the performance of chicken in terms of clinical signs, survival rate, oocysts count, body weight gain and lesion scores, γ-ray attenuation has unequivocally proven much more effective with both the 500 and 1000 oocyst doses when the four efficacy indices (ACI, ROP, POAA and RLS) were evaluated. Therefore, gamma ray attenuation of Eimeria oocysts at 150 Gray especially at 1000 oocyst dose, can be a potential candidate for further study.

Based on the above conclusion, the following recommendations are forwarded.

- ✓ Since, such vaccines are expensive and cannot be easily accessible on local markets, private and governmental institutions should support efforts of developing anticoccidial vaccines locally.
- ✓ Further study is required to test different doses, routes of administration and the shelf life of the product under different temperatures.
- ✓ Since this study was conducted on Eimeria oocysts without species identification, the vaccinal isolates should undergo molecular based identification to make sure against which species is the vaccine more effective.

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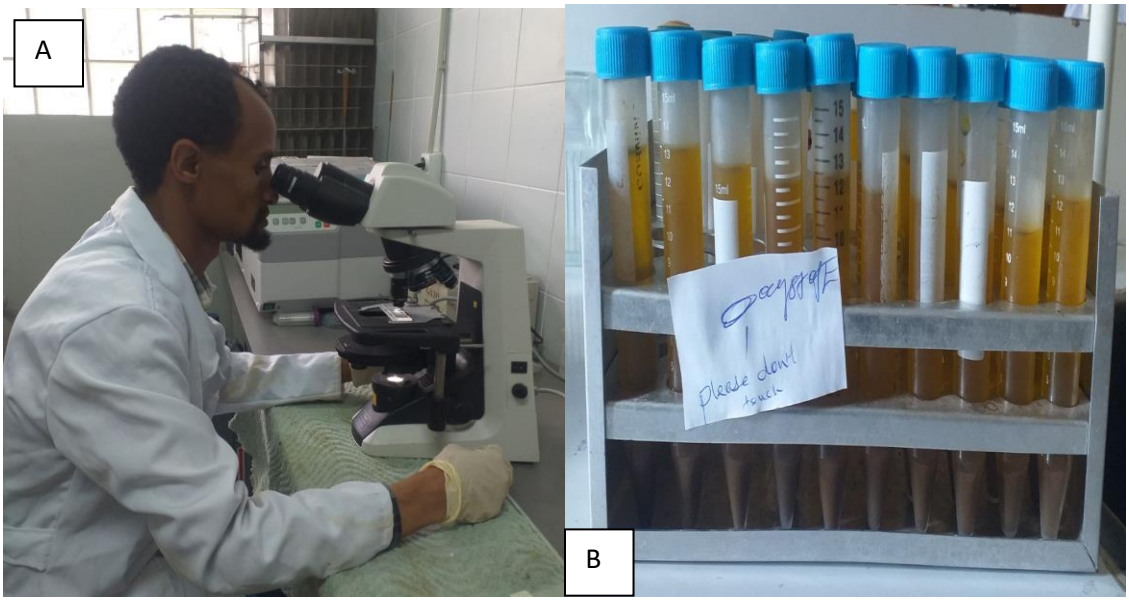
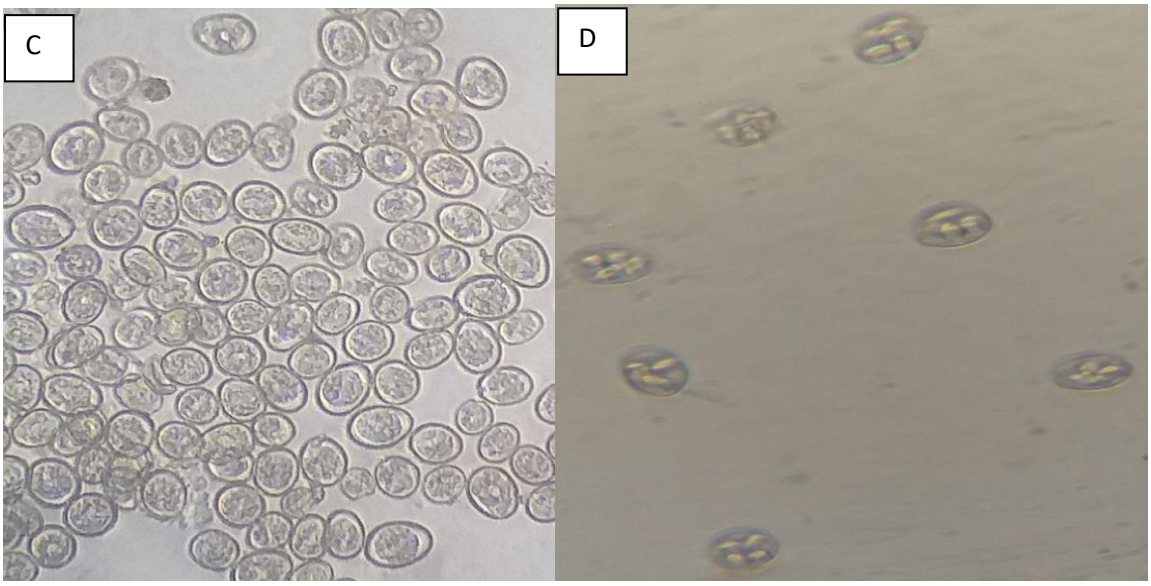
8. ANNEXES

Annex 1: Laboratory examination

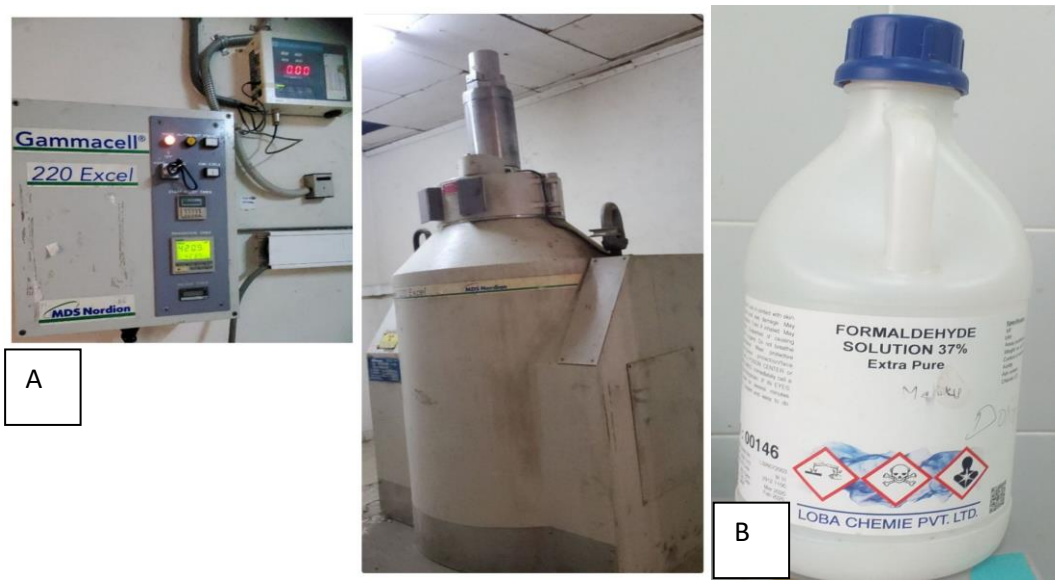


A) Oocyst counting using Macmaster chamber B) Preserved sporulated oocysts



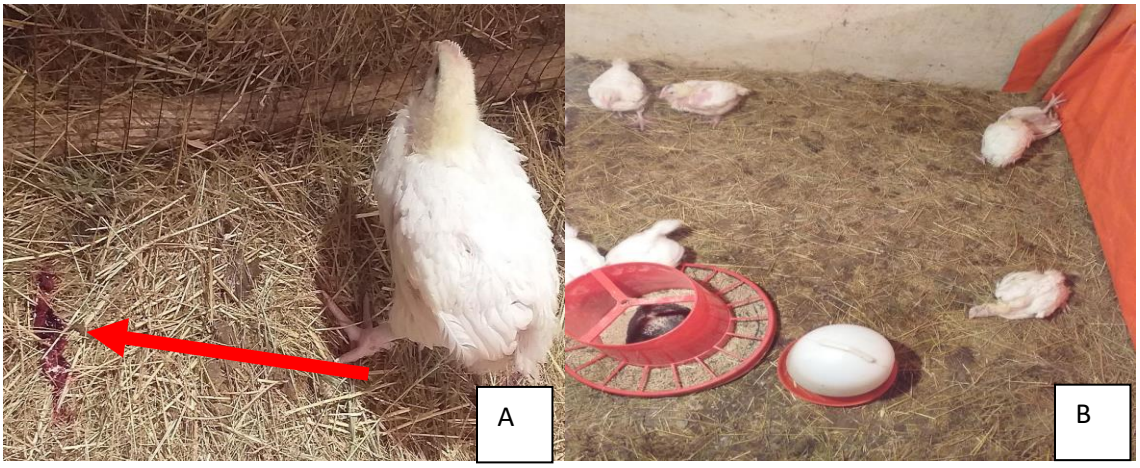
C) Unsporulated oocysts D) sporulated oocysts

Annex 2: Attenuating machine and chemical



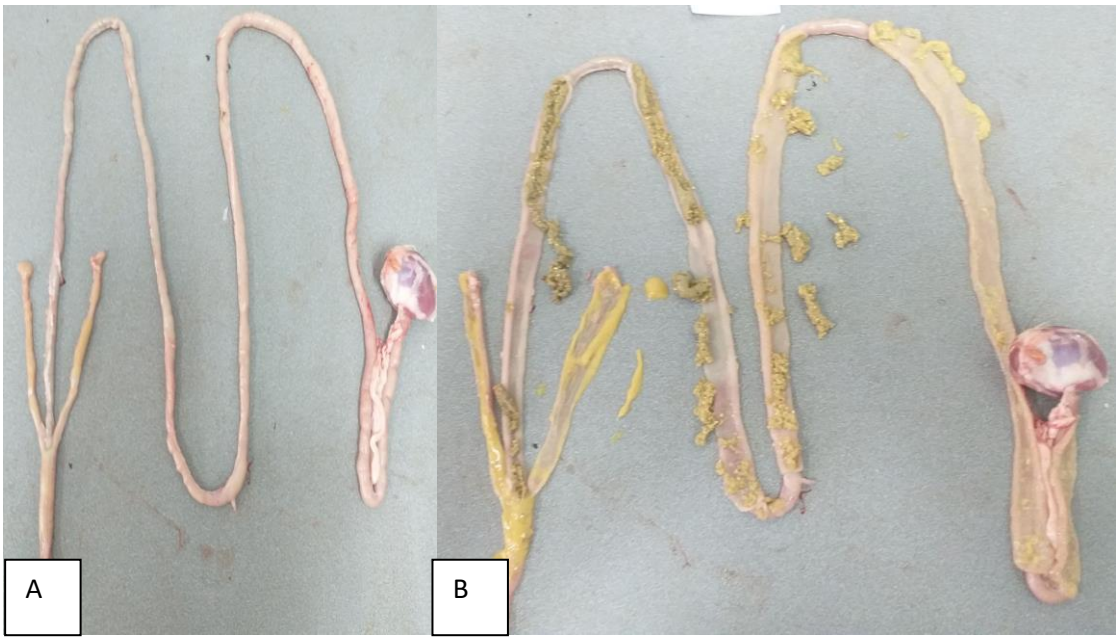
A) Gamma ray machine B) Formalin

Annex 3: Clinical signs of coccidiosis

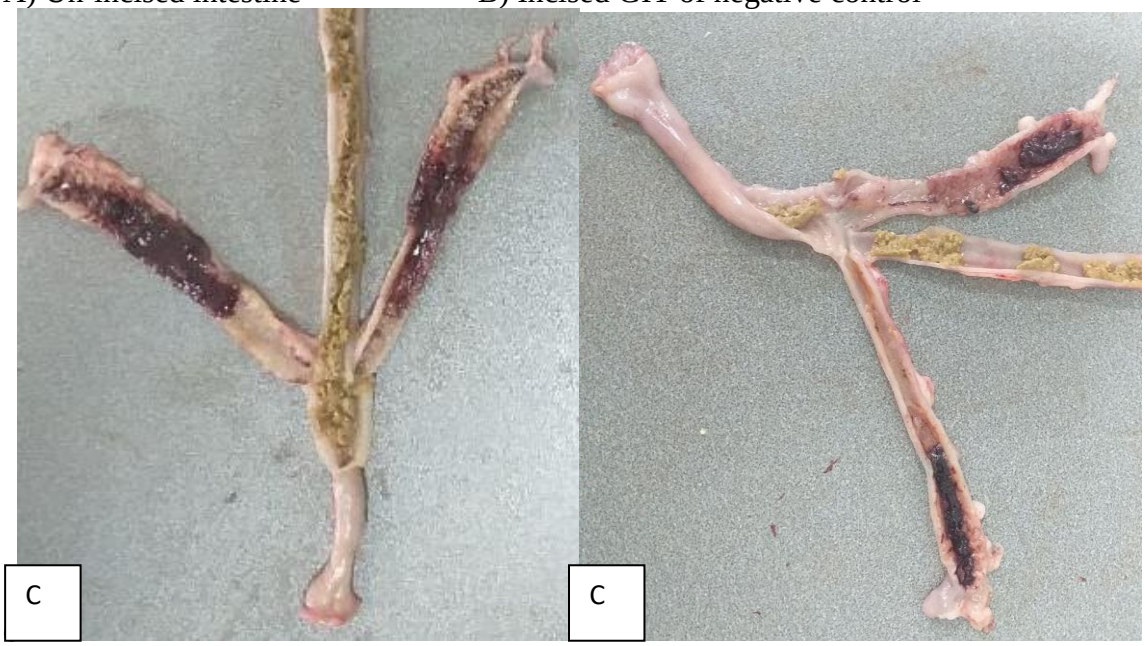


A) Bloody diarrhea B) Depression, ruffled feather with mortality

Annex 4: Post-mortem examination



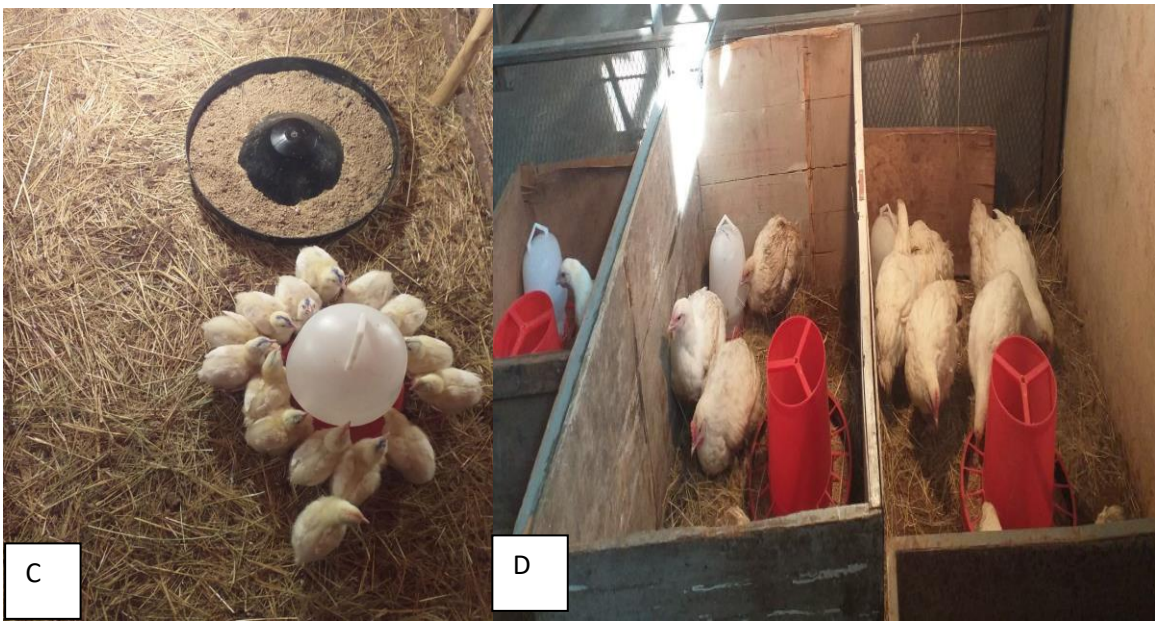
A) Un-incised intestine B) Incised GIT of negative control



C) Thicken and heamorrhagic wall of cecum

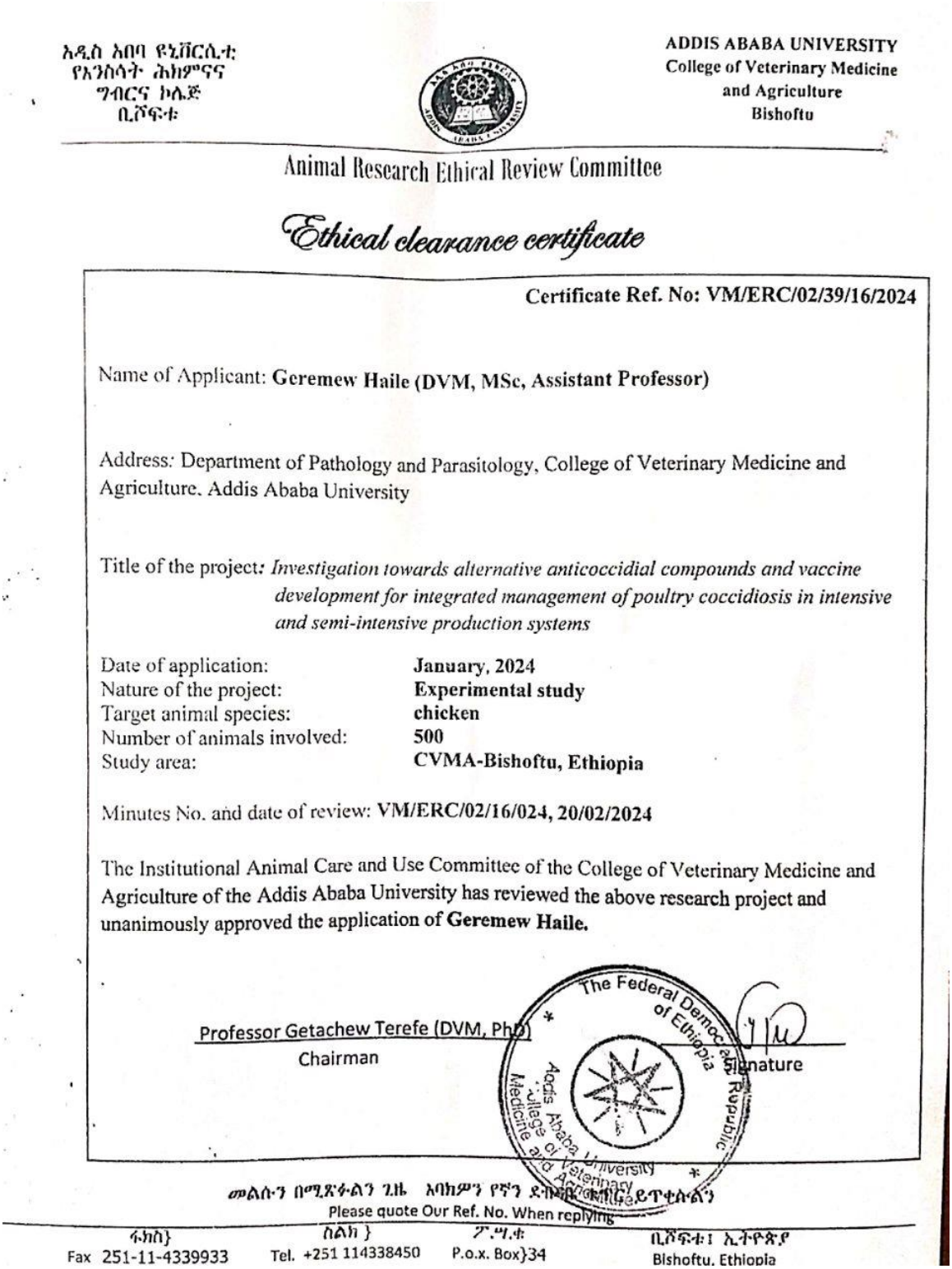
Annex 5: Poultry management practice

A) Grouped day-old chicken B) Vaccination at day 4 old



c) Drinking of vitamin D) Chickens for propagation

Annex 6: Ethical clearance certificate



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ADDIS ABABA UNIVERSITY
College of Veterinary Medicine
and Agriculture
Bishoftu

Animal Research Ethical Review Committee

Ethical clearance certificate

Certificate Ref. No: VM/ERC/02/39/16/2024

Name of Applicant: **Geremew Haile (DVM, MSc, Assistant Professor)**

Address: Department of Pathology and Parasitology, College of Veterinary Medicine and Agriculture, Addis Ababa University

Title of the project: *Investigation towards alternative anticoccidial compounds and vaccine development for integrated management of poultry coccidiosis in intensive and semi-intensive production systems*

Date of application:	**January, 2024**
Nature of the project:	**Experimental study**
Target animal species:	**chicken**
Number of animals involved:	**500**
Study area:	**CVMA-Bishoftu, Ethiopia**

Minutes No. and date of review: **VM/ERC/02/16/024, 20/02/2024**

The Institutional Animal Care and Use Committee of the College of Veterinary Medicine and Agriculture of the Addis Ababa University has reviewed the above research project and unanimously approved the application of **Geremew Haile.**

Professor Getachew Terefe (DVM, PhD)
Chairman

Signature

The Federal Democratic Republic of Ethiopia
Addis Ababa University College of Veterinary Medicine and Agriculture

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EFFICACY OF FORMALIN AND GAMMA RAY ATTENUATED COCCIDIAL VACCINES PRODUCED FROM LOCAL ISOLATES AGAINST CHALLENGE INFECTION IN BROILER CHICKEN

MSc THESIS

BY

MELAKU YISMAW

ABSTRACT

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