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# *IN VITRO* AND *IN VIVO* EFFICACY OF ANTICOCCIDIAL DRUGS AGAINST MIXED *EIMERIA* SPECIES OF CHICKENS COLLECTED FROM POULTRY FARMS IN BISHOFTU TOWN


**MVSc THESIS**

**ADDIS ABABA UNIVERSTIY COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE DEPATMENT OF PATHOLOGY AND PARASITOLOGY**

**BY**

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**JUNE, 2024**

**BISHOFTU, ETHIOPIA**

# *IN VITRO* AND *IN VIVO* EFFICACY OF ANTICOCCIDIAL DRUGS AGAINST MIXED *EIMERIA* SPECIES OF CHICKENS COLLECTED FROM POULTRY FARMS IN BISHOFTU TOWN

**A thesis submitted to the College of Veterinary Medicine and Agriculture, Addis Ababa University in partial fulfillment of the requirements for the Degree of Master of Veterinary Science in Poultry Health and Management**

**By**

**Yemisrach Eyasu Tora**

**June, 2024**

**Bishoftu, Ethiopia**

Addis Ababa University

College of Veterinary Medicine and Agriculture

Department of Pathology and Parasitology

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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: **Yemisrach Eyasu Tora** entitled "***In vitro* and *In vivo* Efficacy of Anticoccidial Drugs Against Mixed *Eimeria* Species of Chickens Collected from Poultry Farms in Bishoftu Town**" and recommend that it be accepted as fulfilling the thesis requirement for the degree of Masters of Veterinary Science in Poultry Health and Management.

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I herby certify that I have read the revised version of this thesis prepared under my direction and recommended that it is accepted as fulfilling the thesis/dissertation requirement.

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## STATEMENT OF AUTHOR

I confirm that the research titled "***In vitro* and *in vivo* efficacy of anticoccidial drugs against mixed *eimeria* species of chickens collected from poultry farms in Bishoftu town"** is entirely my own work, and I have appropriately acknowledged all sources used in this study. This research is being submitted as part of the requirements for an advanced degree of Masters of Veterinary Science at Addis Ababa University, College of Veterinary Medicine and Agriculture, and will be made available in the University's library for borrowing according to library rules. I affirm that this research has not been submitted for the purpose of obtaining any academic degree, diploma, or certificate from any other institution.

Name: Yemisrach Eyasu Tora Signature:_____

Date of Submission:_____

College of Veterinary Medicine and Agriculture, Bishoftu

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| ABW | Average Body Weight |
| ACI | Anticoccidial Index |
| AMPRO | Amprolium |
| ANOVA | Analysis of Variance |
| GSR | Growth and Survival Rate |
| IBD | Infectious Bursal Disease |
| KOKSI | Koksidex |
| LS | Lesion Score |
| NEGC | Negative Control |
| OPG | Oocyst per Gram of Feces |
| POAA | Percent Optimum Anticoccidial Activity |
| POSC | Positive Control |
| RLS | Reduced Lesion Score |
| ROP | Relative Oocyst Production |
| TOLTRA | Toltracox |
| US | United State |

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**ABSTRACT**

Handling of the highly productive exotic breeds of chicken is seriously constrained by widespread diseases, of which coccidiosis poses a persistent challenge. Management of poultry coccidiosis caused by *Eimeria* species is heavily dependent on strict biosecurity practices and use of anticoccidial drugs; drug resistance being a limiting factor. This study, therefore aimed at assessing the efficacy of anticoccidial drugs commonly used in commercial poultry farms in and around Bishoftu. The curative drugs, Amprolium (AMPRO), Toltrazuril (TOLTRA) and Koksidex (KOKSI) made the targets of the study. Oocysts of mixed species were isolated from fecal droppings collected from poultry farms, propagated on chicken and purified for *in vitro* incubation with 28mg/ml AMPROand KOKSI. Sporulation potentials of the oocysts were evaluated against the untreated control. For the *in vivo* study, a randomized control trial was done on 150, day-old Cobb500 breed of broiler chicken. At the end of the $2^{nd}$ week of age,chicken were divided into five treatment groups. Each group was penned in triplicates of 10 chickens (30/group). Accordingly, Groups AMPRO, KOKSI and TOLTRA Received $1.5x10^5$ /chicken sporulated oocysts and then started treatment on the $6^{th}$ day of infection. Group POSC was infected with the same number but left untreated while group NEGC served as a non-infected non-treated control. Clinical observations, body weight measurements, ococyst count, mortality and lesion scores were monitored until day 9 post treatment (PT). Using these parameters efficacy status of the drugs was determined based on four major indices: anticoccidial index (ACI), reduction of oocysts production (ROP), reduction of lesion score (RLS) and percent optimumanticoccidial activity (POAA). The findings revealed: 1) KOKSI had significantly inhibited sporulation of *Eimeria* oocysts, 2) body weight gain was not different among infected groups irrespective of treatment but significantly lower than that of the NEGC (P,0.05), 3) nine days PT, fecal oocysts count was significantly reduced in KOKSI group compared to other infected groups, 4) on day 9PT, survival rate was better with TOLTRA, 5) nine days PT, lesion scores did not significantly differ between treated and untreated groups, 6) with cut off values of 160 for ACI, 15 for ROP, 50 for RLS and 50 for POAA, strong resistance was detected against AMPRO and TOLTRA. On the other hand, with RLS value of 50 (≥50) and ROP value of 0.3 (<15), but failing in the other two indices, KOKSI developed moderate resistance. In conclusion, anticoccidial drug resistance is evident in poultry farms ofBishoftu. It is recommended to pose the use of Amprolium and Toltrazuril, prudently apply Koksidex and strengthen biosecurity protocols to limit health and production impacts of the disease.

**Key words**: *amprolium, efficacy, Eimeria, in vitro, in vivo, koksidex, poultry,toltrazuril*

## 1. INTRODUCTION

Rearing of poultry for different purposes including for meat, eggs and feathers has been a staple of food production since the advent of agriculture (Gržinić *et al.,* 2023). Chickens play a crucial role in the agricultural sector, with more than 70 billion raised in 2020; they contribute significantly to global meat production, accounting for one-third of the total output, and produce over 1.6 trillion eggs for human consumption. As the production of chicken meat and eggs is expected to increase in the coming years, their importance in ensuring global food security and supporting a strong agro-economy will become even more pronounced (Gao *et al*., 2024). Despite these, the commercial poultry sector still faces a number of constraints. Diseases that affect poultry are serious issues that have negative effects on society and the economy. Parasitic diseases are the main cause of possible chronic losses from diseases, both with and without apparent clinical signs. They cause high chicken death and morbidity rates, exorbitant medication expenses, production and market losses and many have the potential for zoonoses (Asfaw *et al*., 2021; Ahmad *et al*., 2024).

Coccidiosis is recognized as the major parasitic disease of poultrythat is caused byprotozoan belonging to phylum Apicomplexa, subclass Coccidia, family Eimeriidae and genus *Eimeria* and it spreads worldwide. *Eimeria* species are among the most important coccidian parasites, infecting mammals, birds and other vertebrates (Abebe and Gugsa, 2018; Ahmed and AlBakri, 2021; Tirfie and Lulie, 2024). Seven species of *Eimeria*, including *E. acervulina, E. brunetti, E. maxima, E. mitis, E. necatrix, E. praecox,* and *E. tenella,* are well known to cause coccidiosis in chickens (Adeyemi *et al.,* 2023).

The most common and pathogenic species that affect the poultry is *E. tenella,* resulting in 100% morbidity and a high mortality (Awais *et al*., 2012). In Ethiopia, *E. acervulina, E. necatrix, E. maxima, and E. tenella* are endemic in all parts of the country and affect mainly young growing birds (Kinung'hi *et al*., 2004).

Coccidiosis of chickens is an absolute intestinal disease characterized by bloody, watery or mucoid diarrhea (Ahmed and AlBakri, 2021). *Eimeria* species are host and site-specific, and this specificity is below the organ level, i.e., they infect only the intestine, and within the intestine, each species has its own preferred region. The *Eimeria* that affects the cecum is the most dangerous one among all types; it often leads to high and rapid onset of mortality (Saeed and Alkheraije, 2023).

The transmission of *Eimeria* parasites to susceptible chicken is through ingestion of feed and water that contaminated with feces containing infective oocysts. These environmentally resistant oocysts can survive in poultry litter for several months. Deep litter rearing system is one husbandry and management technique that promotes oocyst survival and sporulation, which aids in the spread of *Eimeria* (Adeyemi *et al.,* 2023).

Control of the disease has focused on several strategies including: management practices at the farm level, vaccination and anticoccidal drugs (Mesa-Pineda *et al*., 2021). Anticoccidial drugs play a vital role but the emergenceof drug resistance has affected the effectiveness of currently available compounds (Abebe and Gugsa, 2018).Since the 1950s, anticoccidial chemicals have been commonly used for the control of poultry coccidiosis, however, the extensive use of these anticoccidials has resulted in a loss of efficacy of these compounds, triggered by increasing resistance by the parasite (Abbas *et al*., 2011).Due to the limited drugs available on the market and extensive use as a feed additive, the problem is pandemic in many developing countries (Zhang *et al.,* 2023), Hunduma *et al.* (2016) have experimentally demonstrated in chicken reared at the Agricultural Research Center based at Bishoftu (Ethiopia) that coccidian organisms were resistant to Amprolium treatment while they were sensitive to the coccidiostat, Sulfadimidine. However, in this study area, other anticoccidial such as Toltrazuril and Koksidex (combination of Amprolium, Sulfaquinoxaline and Vitamin K3) are also being used under different farm sizes, management and production type. Moreover, for timely intervention and reversal of drug resistance, periodic follow up assessments must be conducted.

Therefore, the general objective of this study was to evaluate the efficacy of commonly used anticoccidial drugs in commercial poultry farms in and around Bishoftu town.

The specific objectives were:

1. To assess commonly used anticoccidial drugs against poultry coccidiosis in and around Bishoftu;
2. To evaluate the efficacy of Toltrazuril, Koksidex (Amprolium+Sulfaquinoxaline+Vitamin K3 combination) and Amprolium against mixed Eimeria infection in experimental chickens;

# 2. LITERATURE REVIEW

## 2.1. Coccidiosis of poultry

### *2.1.1. Etiology and epidemiology*

Poultry coccidiosis is one of the most important parasitic disease which affects intestine of the birds. It is caused by subkingdom protozoan, phylum Apicomplexa, order Eucoccidiorida, family Eimeriidae and genus *Eimeria*. The genus *Eimeria* is obligate intracellular parasite and it is known forits host and site specific(Nahed *et al*., 2022; Pal *et al*., 2023; Souza *et al.*, 2024; Gao *et al.*, 2024; Sharm. and Kim, 2024; Tirfie and Lulie, 2024). There are seven known *Eimeria* species that infect chickens: *E. acervulina, E. brunetti, E. maxima, E. mitis, E. necatrix, E. praecox,* and *E. tenella. Eimeria tenella* is the most common and severe species found in poultry that results high mortality, morbidity, and hemorrhagic lesion (Pawestri *et al*., 2020). A study conducted in India by Jadhav (2023), investigated a new species named *E. tarabaie,* which is clearly distinct from the previously described seven species. The morphology of the oocyst and arrangement of the sporocyst is totally different from the other *Eimeria* species.

Poultry coccidiosis is a worldwide disease but most commonly found in tropical and subtropical areas where environmental and husbandry factors support the oocyst sporulation and spread of the infectious agent (Abebe and Gugsa, 2018; Liao *et al*., 2024). In the Greater Horn of Africa (Djibouti, Eritrea, Ethiopia, Kenya, Somalia, Sudan and Uganda) coccidiosis has ranked within one of the top 20 relevant diseases in poultry (Muñoz-Gómez *et al*., 2024).The disease affects all domestic and wild bird species.Broiler chickens and other birds reared commercially for meat are commonly exposed to conditions conducive to developing the disease.The causative agents of coccidiosis *(Eimeria* species) are found everywhere in poultry farms and they are

resistant to harsh environmental conditions (Shivaramaiah *et al*., 2014; Arafa *et al*., 2023).

Although younger birds are more prone to the infection, both young and old age birds can be susceptible to coccidiosis. During rainy season the prevalence rate of the disease is more, which is due topositivestimulation by the warm and humid weather condition that appreciates sporulation of *Eimeria* oocyst and cause infection in the birds (Onyeabor *et al*., 2023). According to different reports, the prevalence of coccidiosis is more in the intensive farming systems due to high stock density that increases the likelihood of disease persistence (Flores *et al.,* 2022).

### *2.1.2. Life Cycle and Pathogenesis*

The chickens get infection after ingestion of sporulated oocysts that contains four sporocysts, each sporocyst has two sporozoites. The oocyst encysts in the host's small intestine, and the sporozoites contained within the oocyst are liberated. The sporozoites penetrate the epithelial cells (enterocytes), change into trophozoite and multiply asexually. Each generation of asexual reproduction produces multiple schizonts and merozoites. The merozoites are liberated from the cell and infect new cells.It is this stage of the infection that can result in destruction of massive numbers of intestinal cells and unde severe conditions death of the animals (Adjei-Mensah and Atuahene, 2023). .

Following three up to four cycles of schizogony, the final batch of merozoites undergoes single round of sexual reproduction, resulting in the production of gametocytes which later transform to gametes. *Eimeria* species exhibit two distinct stages in their sexual multiplication; the macrogamete and the microgamete. Macrogametes are large cells characterized by polysaccharide granules for energy storage and multiple wall-forming bodies that serve as precursors to the oocyst wall. Microgametes are small, mobile cells containing a pair of flagella that facilitate their movement and fertilization of the macrogamete. The fusion of these gametes gives rise to a zygote enclosed within a

protective wall, which is released in the intestine and eventually expelled as unsporulated oocysts into the environment, marking the beginning of a new cycle (López-Osorio, 2020; Felici *et al.*, 202; Ahad *et al.*, 2023).Typically, when the unsporulated oocyst is passed in the feces, it is not infective because it does not contain sporozoites. After several days or weeks (depending on the species, temperature, humidity and oxygen level) outside of the host's body, the oocyst completes development and sporozoites are found within; this is a sporulated oocyst, and it is infective to the next host. The coccidia life cycle is a continuous process, with reinfection occurring daily.

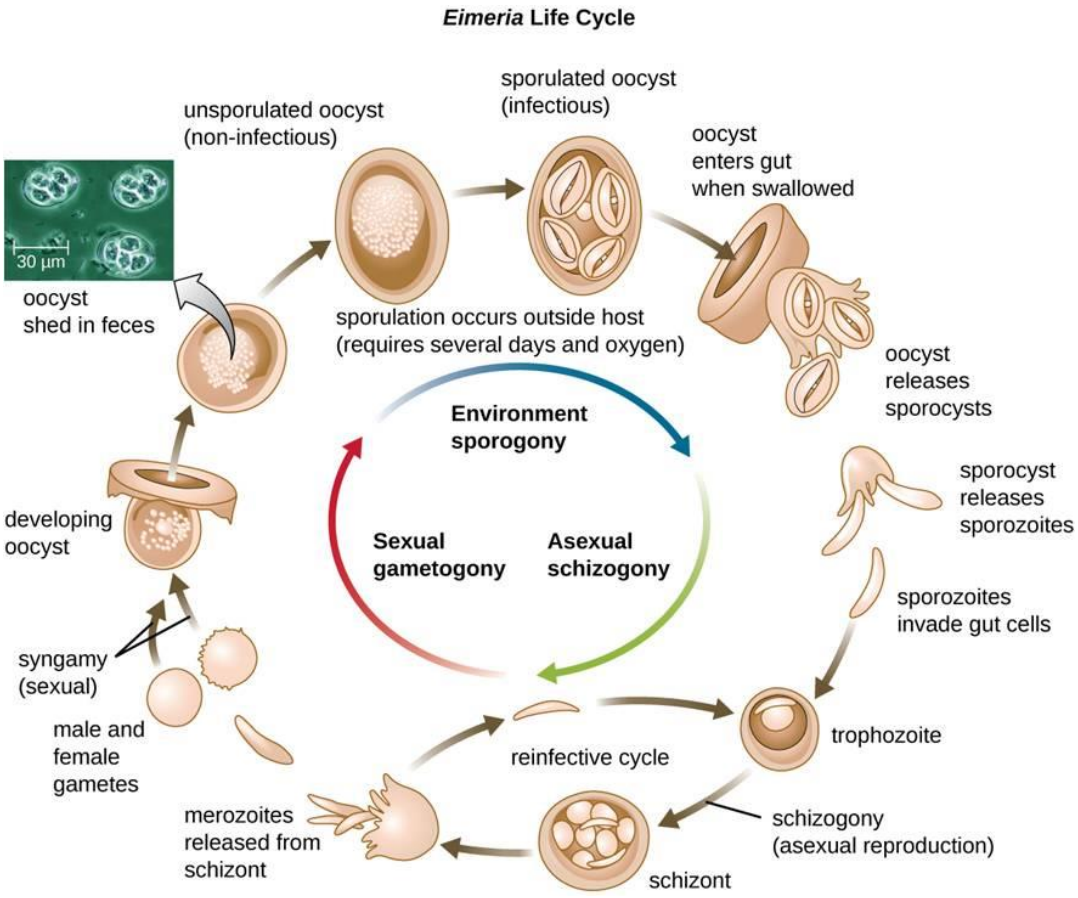


**Figure 1:**Lifecycle of avian coccidiosis*(source: [https://benisonmedia.com/prevention-and-control-of-coccidiosis-in-poultry-production/](https://benisonmedia.com/prevention-and-control-of-coccidiosis-in-poultry-production/) accessed on 23/5/2024*

### *2.1.3. Transmission and clinical signs*

Oocysts are frequent contaminants of litter, feed and water. In a single flock, concurrent infection with at least six species is common (Yu and Heo, 2021). The disease is transmitted among hosts through the fecal-oral route (Arafa *et al*., 2023), by the ingestion of feed, water, and litter contaminated with sporulated oocysts that are shed in the feces of infected animals and spread by fomites or personnel moving between poultry houses. Unsporulated oocyst is excreted from infected birds through feces which sporulate within 48 hours thereby rapidly propagating the spores and the spread of infection (Shivaramaiah *et al*., 2014).It has been shown that the degree of infection and the clinical signs of coccidiosis are influenced by multiple factors including the species of Eimeria, the infective dose and host-parasite interactions.

Sever cases of coccidiosis in chicken are characterized by dysentery, enteritis, emaciation, drooping wings, poor growth, low production with high rate of mortality and morbidity (Awais *et al.,* 2012).The clinical signs observed in coccidia infection in chickens are bloody diarrhea, reduced weight gain, drooping feathers, decreased feed and water consumption and poor growth and reduced egg production. Poor management, such as wet litter that appreciate oocyst sporulation, contaminated drinkers and feeders, bad ventilation, and high stocking density can exacerbate the clinical signs (Gharekhani *et al*., 2014; Santi Devi *et al*., 2019).In post mortem inspection there will be thickening of the intestine, as well as hemorrhage, and enteritis at the specific site of the intestine (Arafa *et al*., 2023).

### *2.1.4. Pathologic effects and lesion scoring*

Coccidia invade the intestinal mucosa and induce a certain degree of epithelial cell damage and inflammation. Meronts, gamonts, and oocysts cause marked histological alterations of host intestinal epithelial cells over a short time period including distortion, rupture, separation from adjacent cells, and sloughing (Yun *et al*., 2000). For the

evaluation of gross lesions, a standardized intestinal lesion scoring technique is used (Johnson and Reid, 1970), which is based on giving a score on a scale of zero to four, with the goal of obtaining a numeric classification of the gross lesions caused by each Eimeria species. For this scoring system, the entire intestine of the bird must be evaluated, beginning with the duodenum. The mucus and serous membranes are examined to detect lesions (Table 1, adapted from Johnson and Reid, 1970).

**Table 1:** Description of gross lesions characteristic of different Eimeria species

| ***Eimeria* species** | **Preferred site** | **Pathogenecity** | **Common lesions** |
|---|---|---|---|
| *E. acervulina* | Duodenum, Jejunum | ++ | Whitish spots on wall on serous surface hemorrhage streak and whitish lesions on intestinal surface, limited mucoid enteritis |
| *E. paracox* | Duodenum, Jejunum | + | No lesion but slightly hemorrhagic appearance on intestinal surface of duodenum, slight mucoid discharge, intestinal watery content |
| *E. mitis* | Ileum | + | Limited enteritis causing loss of fluids |
| *E. maxima* | Jejunum , Ileum | ++ | Distended intestine with hemorrhagic spots, mucoid discharge, swelling of the intestinal wall, detachment of the epithelium |
| *E. brunette* | Cecum, Rectum | +++ | mucoid or necrotic discharge, distended thin walled intestine, swelling of the intestinal wall with hemorrhagic points, detachment of the epithelium |
| *E. tenella* | Cecum | +++ | Severe hemorrhage with white/red spots in wall of cecum, thickening of the walls and blood content in the proximal end. |
| *E. necatrix* | Jejunum, Ileum, Rectum | +++ | Severe hemorrhage with mucoid discharge, whitish and redspot in cecalwall, thickening of the mucosa and cecal lumen filled with liquid orblood, lesions in dead birds are observable as white and black sheets (salt and pepper appearance) |

*+low pathogenicity; ++moderate pathogenicity; +++high pathogenicity*

### *2.1.5. Status of chicken coccidiosis in Ethiopia*

Coccidiosis is highly prevalent and economically significant disease affecting poultry farms in Ethiopia. It is endemic disease in the country and resulting high losses in the sector, particularly in young growing birds of all production systems. In the country most of the time 80% of the disease occurance is recorded as an outbreak (Adem and Ame, 2023). Several studies have reported on the prevalence of coccidiosis from different region of the country. For instance, study by Cheru *et al.* (2023) conducted in East Gojjam Zone of Ethiopia, reported that 10.2% chickens were infected by coccidiosis. Similarly, study conducted by Wondimu *et al.* (2019) documented the overall prevalence of coccidiosis in different intensive farms of Gonder town was 42.2%.

There are also other researches that reported about prevalence of the disease in Ethiopia including present study area (Gari *et al.,* 2008; Alemayehu *et al.,* 2012; Negash *et al*., 2015; Hunduma and Kebede, 2016; Gebeyeh and Yizengaw, 2017; Ketema. and Fasil, 2019 and). *Eimeria* species identified in Ethiopia based on different study *are E. tenella, E. acervulina, E. necatrix, E. maxima, E. mitis, E. brunetti, E. tenella, E. acervulina, E. necatrix and E.mivati* (Lobago *et al*., 2005; Gari *et al.,* 2008; Mulatu *et al*., 2018).

### *2.1.6. Economic important of poultry coccidiosis*

Now a day the world including Africa, have rapidly growing poultry industry with greater potential for development. Between the years 2000 and 2021, a relative change of about 116% to 190%, in chicken meat production has been registered in different parts of Africa (Oke *et al*., 2024). However, huge losses have also been documented due to increased mortality and morbidity of chickens, high biosecurity and treatment costs and decrease in production (Asfaw *et al.,* 2021). Coccidiosis is one of the diseases that commonly encountered in the poultry production worldwide (Wondimu *et al*., 2019).

Coccidiosis has substantial economic importance on the sector because of production losses and costs for treatment or prevention. It might appear as clinical or subclinical

infection, resulting in reduced production with lower weight gain, decreased feed conversion and increased mortality (Gazoni *et al*., 2020). Losses are mainly due to mal-absorption, enteritis and in severe cases for some *Eimeria* species mortality that affects economic productivity and animal welfare (Blake *et al.,* 2020; Abbas *et al*., 2024; Jespersen *et al.,* 2024).

The economic loss because of coccidiosis is common in the whole world but is more prominent in the developing countries, the world losses up to 3 billion US dollars. Only from broiler production, the poultry industry annually gets almost 40 to 42 billion, but coccidiosis causes major losses that reach up to 2.4 billion dollars each year (Abbas *et al*., 2024). A study in India has revealed that commercial broiler industry is a major sufferer due to coccidiosis wherein 95.61 per cent of the total economic loss occurs due to the disease. The commercial layer industry shares 3.53 per cent economic loss, mainly due to cost of chemoprophylaxis and reduced egg production. A comparison across economic traits has revealed that loss is maximumdue to reduced body weight gain, followed by increased feed conversion ratio (23.74%) and chemoprophylaxis (2.83%) in the total loss due to coccidiosis in broiler industry *(*Bera *et al., 2010).*

### *2.1.7. Management of coccidiosis in poultry farms*

Since coccidiosis has such a severe impact on poultry, various control measures have been implemented. The earliest methods used in the control of this disease were strict biosecurity and the use of prophylactics. Biosecurity measures are intended to limit the intake of sporulated oocysts by susceptible individuals so that a subclinical infection is established to induce immunity but not clinical signs (Abebe and Gugsa, 2018). Anticoccidial medications, vaccinations, and stringent management procedures have all been used as a control measure against this parasite (Fatoba and Adeleke, 2018; Mesa-Pineda *et al.,* 2021; Ahmad *et al*., 2024). For a long time, the coccidiosis disease in chicken was successfully controlled by synthetic medications such Amprolium,

Nicarbazin, Diclazuril, and Toltrazuril. The medications specifically target the parasite's sporozoite/merozoite growth stage (Fatoba and Adeleke, 2018).

Prior to the 1930s, the poultry producers had little hope of controlling outbreaks of coccidiosis in growing chickens other than a reliance on flower of sulfur, dry or liquid skimmed milk, and butter milk remedies and the development of a natural immunity through the course of infection. In the third decade of the last century, studies by various workers clearly demonstrated that sulfonamide drugs had significant antibacterial and anticoccidial potential. This pioneering work ultimately led to the discovery, development, and introduction of a wide range of anticoccidial drugs(De Gussem, 2007).

Know a day various types ofanticcocidial drugs are employable to control of poultry coccidiosis. They are classified as synthetic drugs; those produced by chemical synthesis (they are also named “chemicals”), and ionophorous polyether antimicrobials (ionophores) produced by fermentation (Martins *et al*., 2022).To prevent coccidiosis, a variety of coccidiocidal and coccidiostat medications are administered in the feed. While chemical anticoccidial medications have proved successful in treatment of coccidiosis (Saeed and Alkheraije, 2023).Majority of anticoccidial drugs have effectively acted against first and second asexual multiplication sages.

Synthetic substances (chemicas) are Halofuginone, Robenidine, Diclazuril, Decoquinate, Nicarbazine, Toltrazuril, Clopidol, Nequinate, Ethopabate, Amprolium, Sulfadimethoxine, Sulfaquinoxaline. Each synthetic anticoccidial acts in a unique way against coccidia. Because of their chemical synthesis, compounds of this type are often referred to as chemicals and work by interfering with one or more phases of the parasite's life cycle. Once the parasite invades the host's gut, it destroys its intracellular stages. These work well for severe infections, but can lead to increased resistance over the long term due to frequent use (Martins *et al*., 2022).

Most of the earlier synthetic drugs did not completely prevent parasite development and were not completely effective against all *Eimeria* species. Aklomid, Amprolium,

Arprinocid, Dinitolmide, Glycarbylamide, Nicarbazine, Nitrofurazone, Nitrophenid, Polystat, Robenidin, Trithiadol, and Zoalen have been reported not to prevent the development of immunity to various species. In the case of Amprolium and Nicarbazine, several studies suggested that they either delayed or prevented the development of resistance to re-infection (Chapman, 1999).

Ionophores have been the main anticoccidial drugs of choice to treat coccidiosis for many years. They are fermentation products of *Streptomyces* and other fungi species (Okonkwo and Uwalaka, 2020). They are effective against both asexual and sexual life cycle stages of coccidia, and they interfere with the normal movement of ions across the surface membranes of sporozoites or early trophozoites (Noack *et al*., 2019).The mechanism of action of ionophoresis somewhat complicated than others, they do not induce the similar degree of selective pressure on the parasite as synthetic anticoccidials. Because the mechanism of action of ionophores is directed against sporozoites (the parasitic stage in the intestinal lumen prior to host cell penetration) they do not result in total elimination of parasites. Rather, they allow limited amount of parasites in the lumen of the host that helps the birds to develop some degree of immunity (Kadykalo *et al*., 2018).

Until today, more than 120 polyether carboxylic ionophore antibiotics have been identified. From them only six have been selected and are applied as anticoccidials for livestock including poultry. These are Monensin, Lasalocid, Salinomycin, Narasin, Maduramicin, and Semduramicin(Ekinci *et al.,* 2023). Ionophores also act on some gram positive bacteria making them broad spectrum compounds. Also there are some exceptional ionophores that have activity against both gram-positive and gram-negative bacteria (Ekinci *et al.,* 2023).

## 2.2. Anticoccidial drug resistance

Drug resistance is defined as capability of cells or organisms to resist the treatment intervention either intrinsically or in acquired ways (Li, 2013). Drug resistance might be antimicrobial (antibiotic) or antiparsitic. Resistance is said to occur when a pathogenic agent shows any decline in response to drugs and is ended when the maximum amount (dose) of drug that can be tolerated by the host without any result (Sundar*et al.,* 2011; Shalaby *et al*., 2013).The excessive use of antibiotics in the last years causes subsequent negative health and economic impact due to the appearance of resistance of the agent to the drugs. Antiparasitic resistance is the main cause of reduced production and great economic loss in the livestock industry worldwide (Hennessey *et al*., 2020).

Anticoccial drug resistance has been known since the discovery of a sulfonamide resistant strain of *Eimeria tenella* (McDougald, 1981). An abusive and continuous use of anticoccidial drugs have led to the emergence of drug-resistant strains, this limits their use, and is a problem faced with most of the anticoccidial drugs. At the time between 1980s and early 1990s Sulphaquinoxaline, Nitrofurans and Amprolium were the drugs which were commonly used for the treatment of avian coccidiosis.With time, these drugs showed decreasing ability to treat coccidiosis due to an increasing number of resistant *Eimeria* strains in different parts of the world (Usman *et al*., 2011).

A research reported that the *Eimeria* isolate developed resistance to Amprolium. Using Reduced Lesion Score (RLS) values, they found that the *E. tenella* isolate exhibited resistance to Toltrazuril, Clopidol, Salinomycin, and Nicarbazine, and that this isolate exhibited relatively severe resistance to the drugs Salinomycin and Nicarbazine, as measured by the Relative Occyst Production (ROP) values. Because of these serious consequences of resistance, there is an urgent need for new approaches to prevent and control of coccidia infections in chickens (Sun *et al.,* 2023). Hunduma *et al*. (2016) have experimentally demonstrated in chicken reared at the Agricultural Research Center based at Bishoftu (Ethiopia) that coccidian organisms were resistant to Amprolium treatment

while they were sensitive to the coccidiostat, Sulfadimidine. Resistance to anticoccidial drugs can be acquired resistance, cross-resistance or multiple resistance.

Acquired resistance is resistance resulting from an inherited decrease over time in the susceptibility of certain strains and species of *Eimeria* to drugs. For instance, resistance to Sulfaquinoxaline may be induced in *E. acervulina* and *E. tenella*. There are two types of acquired resistance, partial or complete. These types depend upon the extent of sensitivity lost. There is a direct relationship between concentration of the drug and degree of resistance. A strain controlled by one dose of a drug may show resistance when a lower concentration of the same drug is administered. This may allow for the selection of initially resistant mutants to low levels of drug (Abbas *et al.*, 2011)

Cross-resistance is the sharing of resistance among different compounds between the same mechanisms of action (Chapman, 1997).A high degree of cross resistance has been reported among Maduramicin, Monensin, Salinomycin, Narasin, and Lasalocid (Abbas *et al.,* 2011; Peek and Landman, 2011).

Multiple resistance is a resistance of *Eimeria* species to more than one anticoccidial drugs, even though they have different modes of action. In study conducted in Pakistan, researchers detected occurrence of multiple resistance in three field isolates of *E. tenella* obtained from broiler farms (Abbas *et al.,* 2008). There may be many reasons for the development of multiple resistance in field isolates of *Eimeria* strains. Multiple resistance may arise as a result of sequential exposure to the compounds in question in successive flocks. Most likely, genetic recombination is one mechanism by which multiple resistance arise in the field (Stephan *et al.*, 1997). Drug-resistant *Eimeria* strains are responsible for subclinical coccidiosis and, subsequently, for suboptimal economic performance as measured by body weight gain and feed conversion ratio (Györke *et al.,* 2016).

Strategies to reduce resistance to anticoccidial drugs represent a crucial need. Reducing permanent re-infections by improving husbandry conditions in poultry under

anticoccidial prevention or treatment reduces the drug resistance of *Eimeria*. Most poultry producers use rotation, a combination of anticoccidial drugs or even a shuttle program to shift from one chemical to another in the same season to minimize resistance (Khater *et al.*, 2020).

The emergence of resistant *Eimeria* and public concerns about the residues in poultry products led to the poultry producers to use live vaccination against coccidiosis. However, alternative methods are also sought to combat coccidiosis in combination with vaccination. Therefore, various additives such as prebiotics, probiotics, essential oils, and plant extracts have been introduced to the poultry industry in attempts to minimize the negative effects of coccidiosis (Daneshmand *et al.*, 20210).

# 3. MATERIALS AND METHODS

## 3.1. Study area

The experimental study was conducted from November, 2023 to May, 2024 at Addis Ababa University College of Veterinary Medicine and Agriculture in Bishoftu town, Oromia regional state, Ethiopia. Bishoftu is located at the distance of 45km southeast of Addis Ababa. The area is located at $9^0$ N latitude and $40^0$E longitudes with an altitude of 1880 meters above sea level. Questionnaire surveys and fecal sample collection for isolation of *Eimeria* oocysts were done on commercial farms located in and around Bishoftu town. The area has bimodal rainfall pattern with a long rainy season extending from June to September and a short rainy season extending from March to May. The dry season extends from October to February. The temperatures of the area ranges from 14°C to 26°C the humidity is 66% in summer and 56% in winter while the mean annual rain fall is 866mm (Demeke *et al.,* 2023).

## 3.2. Study animals and chicken management

One day old broiler (Cobb 500) chickens were the subjects of the experimental study. A total of 150 day-old broiler Cobb 500 breed of chicks were purchased from ELFORA commercial poultry farm based at Bisoftu. They were kept in cleaned and disinfected animal house which has never been used by chicken. The house is located in the College of Veterinary Medicine and Agriculture premise. They were feed a standard commercial poultry feed without anticoccidial compound supplement. All the chicks were kept on broiler starter ration for 10 days of age and then provided with a grower feed from 11-28 days of age and finisher feed for the remaining days. Water was provided ad libitum. All the birds were vaccinated for Newcastle disease, infectious bursal disease (IBD) and Marek's disease. The chickens were reared in deep litter system. In addition to putting physical barriers, a foot bath was placed at the entrance of the experimental unit to ensure biosecurity protocols. Personal protective clothes including face masks were used by all people in contact with the chicken. Animals were handled by respecting ethical

principles with regards to their welfare and research ethics clearance was obtained from the Institutional Animal Use and Care Committee of the College of Veterinary Medicine and Agriculture of the Addis Ababa University (Ref. No.VM/ERC/03/31/16/2024).

### 3.3.Study design

The experimental study design involved randomized control trial to assess the response of Eimeria to commonly used anticoccidial drugs. Efficacy of three commonly used drugs was tested for their therapeutic effect in experimentally infected chickens. The drugs of test were Amprolium (AMPRO) (Ashampro 20%, Ashish Life Science Pvt Limited, India), Toltrazuril (TOLTRA) (Toltracox 5%, Ashish Life Science Pvt Limited, India) and combination of Amprolium, Sulfaquinoxaline and vitamin K3 (KOKSI) (Koksidex, Medion, Bandug-Indonesia). For each treatment group 30 day-old chicks were divided into triplicate pens. Similarly, an infected and non-treated positive control (POSC) and non-infected and non-treated negative control (NEGC) groups were formed with similar numbers of chicken.

### 3.4.Questionnaire survey

Along the fecal sampling, a questionnaire surveys was administered to farms, and local pharmaceutical suppliers to know which anticoccidial drugs are commonly used in the study area. Wata were collected from 62 commercial poultry farms in and around Bishoftu. Moreover, 20 veterinary pharmacies visited to gather information regarding common anticoccidial drugs available and the choices of customers as reflected by the demand.

### 3.5.Farm fecal sample collection as source of coccidia oocysts

Several farms were visited to selected potential sources for infected chicken. Up on permission from suspected farms, fecal samples were collected from clinically sick young

chickens (aged 15- 30 days old) and transported to Parasitology laboratory of the College of Veterinary Medicine and Agriculture at Bishoftu. Simple floatation technique was first employed to confirm presence of coccidian oocysts and the Macmaster technique was employed to estimate oocysts per gram of fecal material. Then, samples with good number of oocysts were pooled and preserved at +4ºC until further purification.

### 3.6.Oocysts purification and sporulation

Oocysts in the pooled fecal samples described above were purified by the method outlined by Smith and Ruff (1975). Briefly, the fecal material was strained through cheese cloth into a large beaker with several washings of water. The fecal suspension was thoroughly mixed and allowed to stand. When a ring of denser debris formed near the bottom of the beaker, the supernatant was decanted off and saved. This supernatant contained oocysts as well as smaller fecal particles. To extract larger quantities of oocysts from the original sediment, repeated resuspension and settling of the sediment was performed. The collected supernatant was then centrifuged for 5 minutes. This time, the sediment after centrifugation was saved and mixed with 2.5% potassium dichromate (K2Cr2O7) while the supernatant was discarded. The mixture was kept at room temperature for nine days with frequent shaking and aeration to allow maximum sporulation. Then oocysts number was counted by modified McMaster technique (Zajac *et al*., 2014) but to determined sporulation percentage under the microscope, Neubauerhaemocytometer (Abunemeh, 2016) was employed for better visualization at 40x magnification. On the $9^{th}$ day of incubation about 85% of sporulation was reached. The suspension was tightly stoppered and stored at +4° C until further use.

### 3.7. Donor chick infection and propagation of coccidial oocysts

Six healthy Sasso breed of chickens aged 20-25 days were purchased from Bishoftu local market, with the aim of propagating oocystsfor future use in the main experiment. Each chicken was experimentally drenched with a graduated pasture pipette at an infection dose of $1\times 10^5$ oocysts (85% sporulated) obtained in the above process. Following

development ofinfection,as much fecal sample as possible was collected between four and 8 days post infection. The samples went through the purification and sporulation processes described above and the numbers of oocysts/ml of suspicion and the proportion of sporulation was quantified. Finally, 2.5% potassium dichromate was added and the material was stored at +4ºC for further use.

### 3.8. *In vitro* anticoccidial drug efficacy test

This test is intended to assess the potential of each drug to limit sporulation potential of Eimeria oocyts at room temperature. Three anticoccidial drugs were chosen based on their extensive use in the study area as per the preliminary survey conducted among veterinary drug retail shops in Bishoftu. The initial plan was to test, all the three drugs (AMPRO, TOLTRA and KOKSI). However, TOLTRA proved to form a kind of gel preventing successive sampling for evaluating sporulation rate. The in vitro tests were conducted in two different phases. First, before the in vivo study on chickens, the effect of AMPRO and KOKSI was evaluated in vitro. About 45,000 unsporulated oocysts in 300µl water suspension was exposed to 28mg/ml of AMPRO and 28mg/ml of KOKSI as described by Murshed et al. (2023). The anticoccidial dilution has taken into account the volume of water in which the oocysts were suspended. The mixture was filled in 3ml Eppendorf tubes in six replicates. Another set of tubes was filled with similar volume of oocyst suspension but without treatment, forming a negative control group. The tubes were kept at room temperature for four days with frequent shaking and aeration. Aliquots of 10µl/tube were taken every day to see the levels of sporulation under light microscope at 40x magnification. Oocyst with visible four sporocysts was considered as sporulated.

In the second phase, oocyst containing fecal samples collected from chicken treated with the three anticocidial drugs were incubated at room temperature for three days to see if treatment reduces sporulation potential of oocysts in the environment. In both cases, since it was difficult to differentiate at species level on the basis of oocyst morphology, the sample materials were assumed to contain a mix of pathogenic *Eimeria* species. The

percent sporulation of *Eimeria* species was determined as follows from the total oocysts counted by using Neubauerhaemocytometer:

$$\text{Sporulation\%} = \frac{\text{Number of sporulatedoocysts}}{\text{Total number of oocysts}} \times 100$$

### 3.9.*In vivo* drug efficacy test in chicken

#### *3.9.1. Pilot infection dose determination*

Coccidial infections are self-limiting and depend largely on the number of sporulated oocysts ingested. There is an optimal dose, such that the reproductive potential of the parasite is met and they efficiently replicate within the epithelial cells (Cervantes *et al.*, 2020). For the *in vivo* experimental study, infection dose was first tested at three different doses. For this, 15day-old cobb500 broiler chicks which were generously donated by the ELFORA broiler farm were divided into three treatment dose groups (5chickens/dose) and reared under coccidia-free environment. Group I was drenched with $1x10^5$oocysts, group II with $1.5x\ 10^5$ and group III with $2x\ 10^5$ oocysts/chicken. Oocysts outputs were counted starting from day 4 post infection while observations on clinical features started on the second day of the infection. The result showed that the first dose was inadequate while the highest dose was excessive and caused mortality in early days of infection. All infected animals have shown diarrhea but this was severe and bloody in the two higher doses. Therefore $1.5x\ 10^5$ Oocysts/chicken was chosen for the actual experimental infection.

#### *3.9.2. Experimental infection and treatment of chickens*

One hundred fifty day-old Cobb 500 broiler chickens were purchased from ELFORA Poultry farm PLC and reared together in a separate animal unit until the age of 12. Then, their body weights were measured on $14^{th}$ day of age (Day -6) followed by random allocation to each treatment group in their respective triplicate pens after adjusting for

body weight. Chicken in groups AMPRO, KOKSI, TOLTRA, and POSC were drenched on the same day with 150,000 oocysts (85% sporulated) per chicken in 2ml distilled water. The negative control group (NEGC) received 2ml of distilled water as a vehicle control. Following detection of oocysts in feces of all infected groups, groups AMPRO, KOKSI, TOLTRA started to receive treatments six days post infection (Day 0, age: 20 days old) with Amprolium (powder) at the rate of 5g/4 L drinking water (Ashish Life Science Pvt Limited, India), Amprolium+Sulfaquinoxaline+vitamin K3 (powder) at1g/L of water (Medion, Bandug-Indonesia), and Toltrazuril (liquid) at 7mg/kg with drinking water (Ashish Life Science Pvt Limited India). Treatment continued for 2-8 days depending on the type of anticoccidial drug and as recommended by the manufacturers. Groups POSC and NEGC remained untreated (Table 2).

**Table 2**: Experimental animal infection and treatment schedule

| Groups | No. of replicate | Chicken/ replicate | Chicke/ group | Oocyst/ chicken (Day -6) | treatment schedule | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | D 0 | D1 | D2 | D3 | D4 | D5 | D6 | D7 | D8 |
| AMPRO | 3 | 10 | 30 | $1.5x10^5$ | X | X | X | X | X | | | | |
| KOKSI | 3 | 10 | 30 | $1.5x10^5$ | X | X | X | | | X | X | X | |
| TOLTRA | 3 | 10 | 30 | $1.5x10^5$ | X | X | | | | | | X | X |
| POSC | 3 | 10 | 30 | $1.5x10^5$ | - | | | | | | | | |
| NEGC | 3 | 10 | 30 | 2ml distilled water | - | | | | | | | | |

*3.9.3. Clinical observation*

Chickens were observed twice a day for any clinical signs (feathering characteristics, abnormal excreta, feeding and drinking, etc) and behavioral changes as well as to collect any dead birds for necropsy.

### *3.9.4. Body weight*

Body weight measurements in grams were done using digital sensitive on the day of oocysts drenching (Day 0PI), on the day of first treatment (Day 0PT), that is 6 days post infection, and the last measurement was day 9 post first treatment. In each group, average weight for each pen was registered. From the body weight data, the following values were calculated to aid evaluation of anticoccidial efficacy/resistance based on Lan *et al*. (2017):

$$\text{Relative rate of body weight gain (RRBWG)} = \frac{\%\text{ change in weight gain of treatmentgroup}}{\%\text{ change in wt of Positive control group}}$$

$$\text{Growth and survival rate (GSR)} = \frac{\text{Final body weight (Day 9PT)}}{\text{Initial body weight (Day 0PT)}}$$

### *3.9.5. Fecal examination and occyst count*

Fecal oocysts counts were done using floatation technique described by Ghaniei *et al.* (2022). After filtration and floatation steps, the McMaster egg counting technique was employed where oocysts in both chambers were counted and values were multiplied by 50 to get oocyts per gram of feces (OPG). Then the relative oocyst production (ROP) values were calculated to aid determination of anticoccidial efficacy as follows (Lan *et al*., 2017):

$$\text{ROP} = \frac{\text{OPG of Treatment group}}{\text{OPG of POSC}} \times 100$$

### *3.9.6. Necropsy and intestinal lesion scoring*

Scheduled necropsy examination was conducted three times during the study period. The first was done at six days post inoculation, immediately before treatment, the second was on fifth day post treatment and the last was conducted on the ninth day post treatment. All the chickens sacrificed were selected randomly from each group. The initial postmortem examination was done on two chickens per group while successive euthanasia was conducted on single representative animals per group. Animals were sacrificed by the cervical dislocation method. This was followed by opening of the abdomen and longitudinal incision of the intestines starting from the rectum to the level of the duodenum. Lesion scoring was done on the different regions of the intestine as described by Johnson and Reid (1970) and Conway (1979). Serosal color and texture, mucosal content and thickness, presence of blood, change in the color and consistency of luminal content, etc were considered in the assessment. Based on the severity of the lesions**,** the scores were classified into grades as 0-4. Dead birds were considered to fall in grade 4. The relative Lesion scores (RLS) were calculated as described in Lan *et al* (2017).

### *3.9.7. Determination of chicken survival rate*

Some species of coccidia cause significant mortality in the field. Mortality was recorded starting from the day of infection up until the end of the experiment (day 9 post treatment). Data were used to calculate chicken survival rate with or without anticoccidial treatment. As follows (Ojimelukwe *et al*., 2018):

$$\text{Survival rate} = \frac{\text{Number survived at Day 9PT}}{\text{Number at Day 0PT)}} \times 100$$

### 3.10. Determination of Anticoccidial drug Efficacy/Resistance

Studies have demonstrated that the fecal egg count reduction test recommended by Coles *et al.* (1992) cannot correctly assess efficacy or determine resistance status of anticoccidial drugs. Therefore, a four parameter evaluation used by Lan *et al* (2017) where four assessment indices: Anticoccidial Index (ACI), Relative Oocyst Production (ROP), Percent Optimum Anticoccidial Activity (POAA) and Reduction of Lesion Score (RLS) were calculated from body weight measurements, chicken survival rate, lesion scores (LS), oocyst values, and growth and survival rate (GSR) as follows:

i. ACI = (rate of relative body weight gain + survival rate) – (lesion score + oocyst value).

   An ACI value of $\geq 160$ indicates sensitivity while a value $< 160$ indicated resistance.

ii. ROP = $\frac{\text{Mean oocyst output of treatment group}}{\text{Mean oocyst output of POSC}} \times 100$

If ROP was $\geq 15\%$, it was judged to be resistant and if $<15\%$ it was considered sensitive

iii. POAA = $\frac{\text{GSR in treatment group} - \text{GSR in POSC}}{\text{GSR in POSC} - \text{GSR in NEGC)}} \times 100$

Where GSR (Growth and Survival Ratio) was defined as final body weight divided by initial body weight. POAA $> 50\%$ was judged to be sensitive and $\leq 50\%$ was taken as resistance.

iv. RLS= $\frac{\text{Average LS of POSC-Average LS of Treatment}}{\text{Average LS of POSC}} \times 100$

An RLS value of $\geq 50$ indicates sensitivity while a value $< 50$ indicated resistance.

Overall judgment: if all or three of the values fall in the resistance category, it is considered as strong resistance, if two of the four fail to meet the minimum point, it

shows moderate resistance and one shows slight resistance to the specific drug under consideration (Lan *et al*., 2017).

### 3.11. Data management and analysis

The datawas entered in to excel spread sheetand imported in to STATA version 14 for analysis. Data was analyzedin one-way ANOVA and used bonferonni multiple comparison test to compare the difference of mean between and within groupsto determine the variation. The difference was considered significant if $P < 0.05$.

## 4. RESULTS

### 4.1.Anticoccidial drugs demand and preference in the study area

This questionnaire survey was conducted to collect information about anticoccidial drug usage, and commonly available anticoccidial drugs on the local market. From the total of 62 poultry farm owners who participated in the interview, half of them responded that their chickens have encountered disease conditions with characteristic signs of coccidia infection at least once and reported that they used anticoccidial drugs to manage the problem. The drug most preferred by the respondents was Ashampro (Amprolium) for 16 (25.81%), Koksidex for 9 (14.52 %) them and Toltrazuril for 6 (9.68%) of them. The 20 veterinary pharmacies/drug vendors interviewed have also unanimously confirmed farm owners' order of preference based on the level of demand for the three drugs (Table 3). Unfortunately, none of the poultry feed processors visited was willing to disclose if the feed they produce was supplemented with coccidiostates and the type of product added.

**Table 3:**Aticoccidial drugs with high demand from the local veterinary drug shops

| Most demanded anticoccidia drug | Frequency | Percent |
|---|---|---|
| AMPRO | 11 | 55.00 |
| TOLTRA | 6 | 30.00 |
| KOKSI | 3 | 15.00 |
| Total= | 20 | |

### 4.2.*In vitro* anticoccidial drug efficacy

The present *in vitro* study was planned to evaluate efficacy of two anticoccidial drugs, which are highly demanded by the poultry farm owners and available in the study area, on mixed *Eimeria* species. The result indicated that the sporulation percentage of *Eimeria* exposed to 28mg/ml of AMPRO was 43.32 % after 24 hours, 63.9 % after 48, 64.5 % after 72 and 78.34 % after 96 hours of incubation in the presence of 2.5 % potassium dichromate. Percent sporulation recorded for KOKSI (28mg/ml) was 1.7 %, 5.5%, 38.68 and 52.24 % at 24, 48, 72 and 96 hours post exposure respectively. The *Eimeria* oocysts incubated in 2.5 % potassium dichromate (Control) without any of the two treatments have sporulated at the rate of 50.7 % after 24, 67.6 % after 48, 70.1 % after 72 and 82.2 % after 96 hours. The variation of sporulation percentage between the control and Amprolium groups was not statically significant (P>0.05). However, Koxidex has significantly inhibited sporulation on the 48$^{th}$ and 72$^{nd}$ hour of incubation (P<0.05).

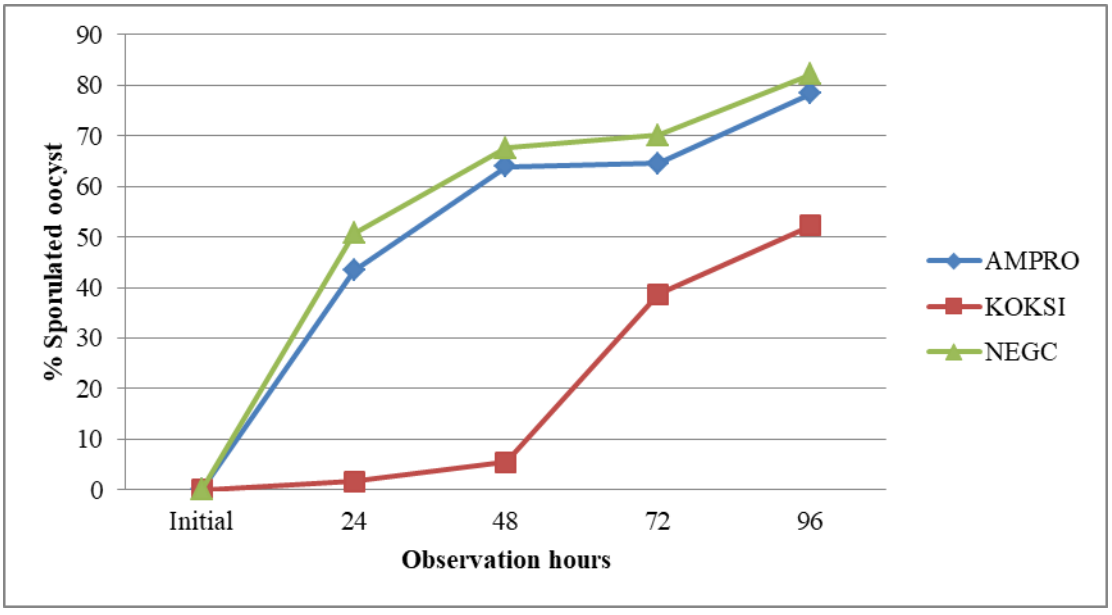


**Figure 2**: Percentage sporulation of occysts incubated with Amprolium and Koksidex compared to the non-treated wells

## 4.3. Anticoccidial drug efficacy in chickens

### *4.3.1. Development of clinical coccidiosis*

Except for one chicken that has shown signs of ascites and died before the start of treatment in other groups, none of the chickens in the non-infected control group have shown signs of abonormal health conditions characteristic of coccidiosis. On the other hand, chickens in the infected groups have started showing clinical signs of ill health three days post inoculation. The most obvious signs were depression, decreased feed and water intake and ruffled feathers. Starting from the 4$^{th}$ day of infection most chicken started to show bloody, mucoid or watery diarrhea until the day of treatment (6$^{th}$ day PI) and this continued for the next 24 hours after treatment i.e. 7$^{th}$ day of infection (Figure 3). These clinical signs have shown improvement in the subsequent days following treatment in treated groups (AMPRO, KOKSI and TOLTRA) but such changes were somewhat delayed in infected non-treated positive control group (POSC).


**Figure 3:**Clinical signs observed in infected group of chicken:

A) bloody fecal material, B) watery diarrhea, C) depressed chicken with ruffled feathers

### *4.3.2. Body weight changes in experimentally infected and treated chicken*

The efficacy of Amprolium (AMPRO), Koksidex (KOKSI) and Toltrazuril (TOLTRA) against chicken coccidiosis in different groups of Cobb500 broiler chickens was evaluated (Figure 4). Weight measurements were done at four different time periods: on the day of infection (Day 0PI), on the day of first treatment (Day 0PT), five days post treatment (Day 5PT) and at the end of the last treatment (Day 9PT). Overall, all groups have demonstrated steady increase in body weights. Since chicken groups were blocked based on body weight, the mean body weight at the start of the experiment ranged between 172 and 177 grams and there was no difference. Six days after infection, at the start of the first treatment, mean body weights were 262±12.4, 268±1.1, 275±2.7, 266±11.9 and 292±9, respectively for group AMPRO, KOKSI, TOLTRA, POSC and NEGC. Although values for the negative control group (tend to be higher, the difference among the groups was not significant ($P>0.05$). Five days post treatment (Day 5PT), infection has caused significant reduction ($P<0.05$) in growth rate compared to the negative control (NEGC) with mean body weights of 314±21.3, 332.±22.0, 333±7.8, 350.0±12.3 and 385±4.8 recorded for the five groups respectively. On the other hand, no difference was observed among infected and then treated groups (AMPRO, KOKSI, TOLTRA) or between these and the non-treated positive control group (POSC). A similar situation was noticed on the 9th day of the start of treatment regimen, mean body weightof the NEGC group being higher compared to values for treated groups and the POSC group.

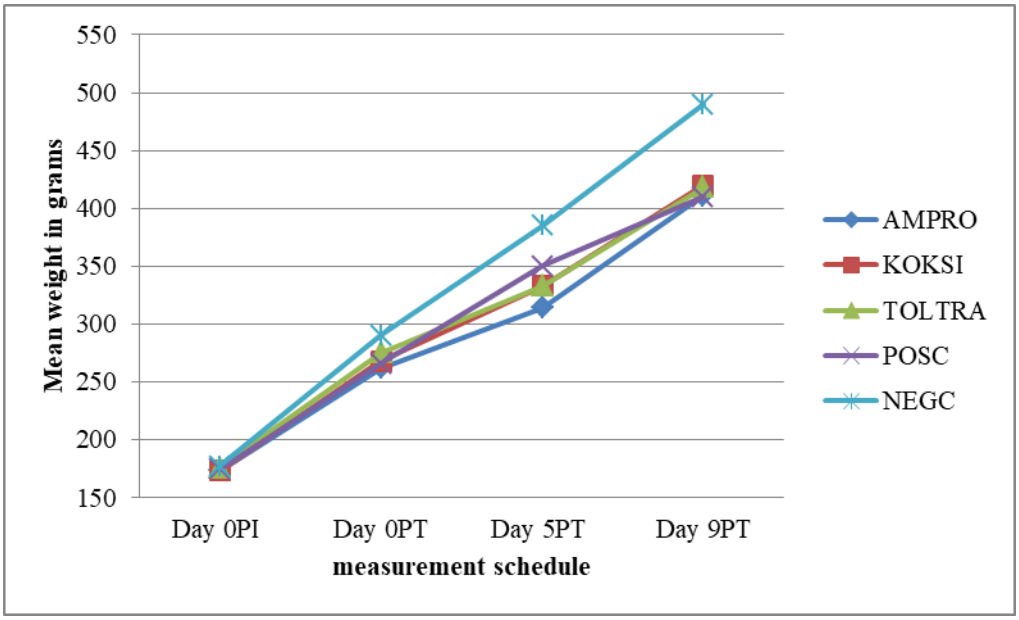


**Figure 4**: Body weight in grams of experimental chicken

*4.3.3. Effect of treatment on fecal oocysts count*

No oocysts were detected in the non-infected control group throughout the experimental period. Following oocyst gavage on their 14$^{th}$ day of age, *Emiria* oocysts start to appear in feces of infected groups on the 4$^{th}$ day post infection. The oocyst count exponentially increased in the next two days (Table 4) at which decision was made for starting treatment. As all groups were in triplicate pens, median counts were taken for each group at all oocyst counting schedule. Since there was big variation in oocyst count on the day of treatment (Baseline value at Day 0PT), successive values were calculated as percentages of the baseline value (baseline value-value at each counting point x100). Accordingly, the count at Day 0PT being 100% (Table 4), the fecal oocysts count was presented in (Figure 5) below. It can clearly be understood that, in all treatment groups, OPG count dramatically dropped two days PT (7 days post infection) including for the non-treated control. However, successive daily counts until Day 5PT revealed significant difference between the non-treated control (23-25%) and the treated groups; 9-3% for AMPRO (P=0.024), less than 1% for KOKSI (P= 0.009) and 5-3% for TOLTRA (P= 0.024). This implies a reduction of about 75 to 77% in the control, 91-97% in AMPRO,

99% in KOKSI and 95-97% in TOLTRA groups. On the other hand, nine days PT, KOKSI performed best with significant reduction compared to both the positive control (POSC) and the other treatment groups (AMPRO and TOLTRA) while the latter two did not significantly differ from the POSC.

**Table 4**: Median oocysts count before administration of treatment

| | Day 5I | Day 0T |
|---|---|---|
| AMPRO | 424000 | 889000 |
| KOKSI | 451600 | 660000 |
| TOLTRA | 630600 | 537700 |
| POSC | 651400 | 944100 |
| NEGC | 0 | 0 |

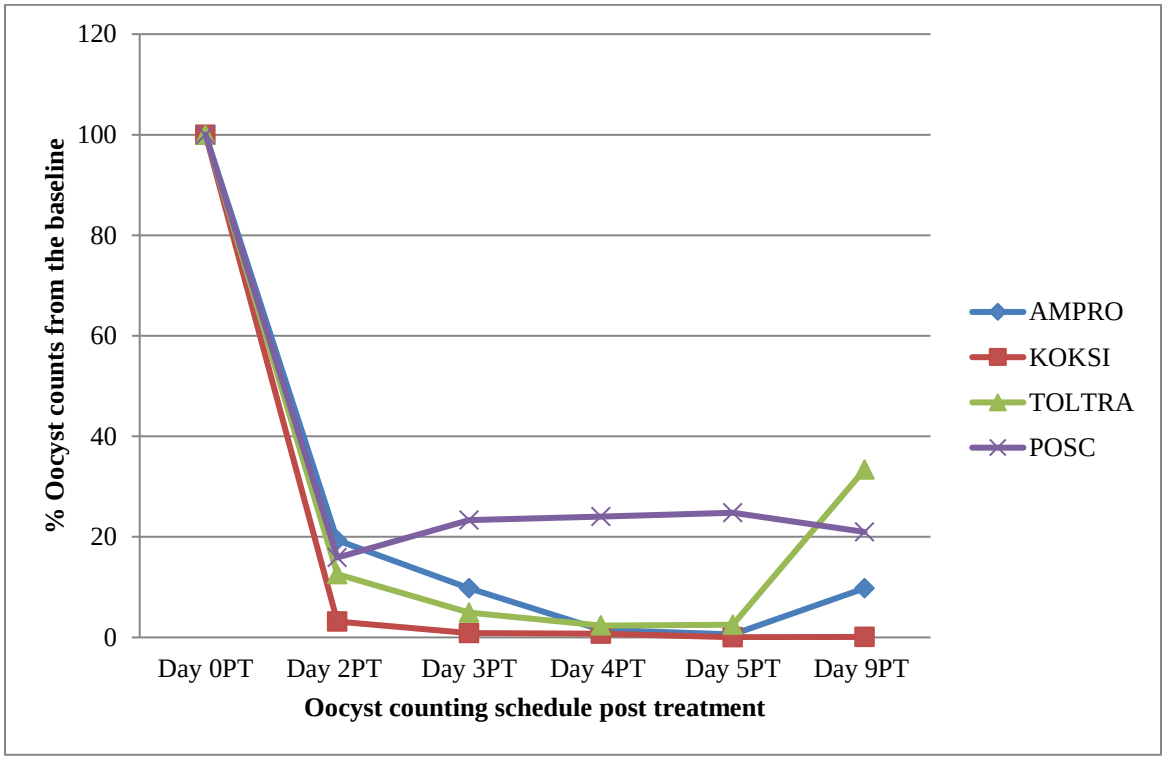


***Figure 5**: Fecal oocyst count expressed as percentage of values at Day 0PI*

### *4.3.4. Coccidia Infected Chicken Survival Rate With and Without Treatment*

On the 6th day of infection, just before treatment was started, the survival rate (among 30 chickens per group) ranged between 93% and 97% in infected groups but no animal died in the negative control group. Following treatment of surviving animals, survival rate continue to decline until 6th day post treatment in groups POSC, KOKSI and AMPRO with 69%, 78.6% and 79.3% respectively (Figure 6). On the other hand, at this stage of treatment, survival values for the NEGC and TOLTRA groups was 100% and 93% with significant difference between the two clusters ($P< 0.05$).

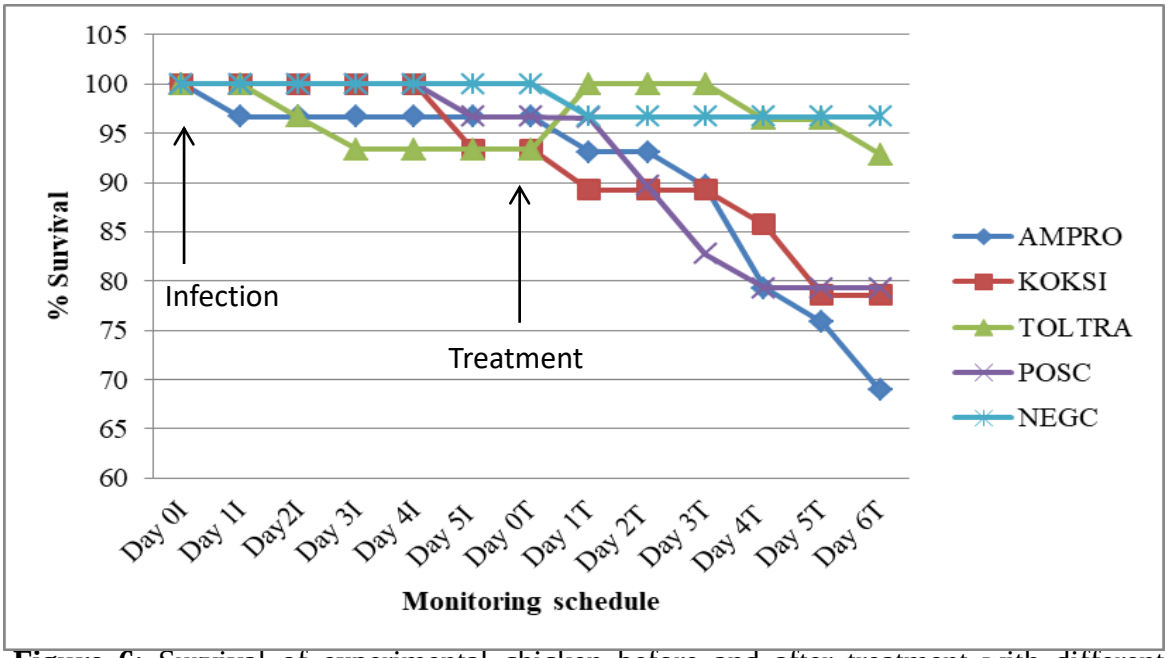


**Figure 6**: Survival of experimental chicken before and after treatment with different anticoccidial drugs

### *4.3.5. Effect of Treatment on Postmortem Lesion Scores*

Since chicken were drenched with parasite suspension supposed to contain mixed species of *Eimeria*, it was expected that lesions may appear at different levels of the small and large intestines. Therefore, the intestines were opened step by step from the rectum up until the proximal part of the duodenum. Accordingly, serosal wall color, intestinal size and thickness, mucosal surface and mucosal contents were carefully inspected and

changes were recorded to classify lesions into grades 0-4. Intestines of all chicken taken from the negative control group (NEGC) were categorized under grade 0 as no abnormality was detected in the intestines.

Mild changes in the wall of the intestines of infected chicken such as presence of scattered white spots or small petechiae were designated as grade 1. This was seen particularly in the later stage following treatment. Similarly, more petechial hemorrhages with slight thickening in the ceca or ladder like lesions in the duodenum and jejunum but with normal intestinal content were taken as characteristic of lesion score 2. This was also common, in chicken killed after the start of treatment with anticoccidial drugs. On the other hand, score 3 and 4 lesions were observed on the $5^{th}$ and $6^{th}$ day of infection before the start of anticoccidial treatment. Score 3 was characterized by coalescent ladder like lesions completely covering the mucosa coupled with thickened wall with watery content in the duodenum and jejunum or presence of large amounts of blood in the cecum with greatly thickened caecal walls and little, if any, fecal contents. Score 4 duodenum and jejunum were seen as havingcoalescent lesions with white greyish color, wall thickened with creamy exudate while ceca in the same score were greatly distended with dark blood, large caseous cores or even without any content. In all cases, dead animals were considered to fall in score 4 (Figure 8). For analytical purposes, mean lesion scores were calculated for each group and presented in (Figure 8). It can be observed although mean lesion score has significantly decreased in the non-treated positive control group, treatment with Koksidex and Toltrazuril has significantly decreased intestinal damage compared to the positive control at Day 9PT. on the other hand, lesions created before treatment appear to have persisted until Day 5PT as the scores were not significantly different from the Day 0PT (day of first treatment).

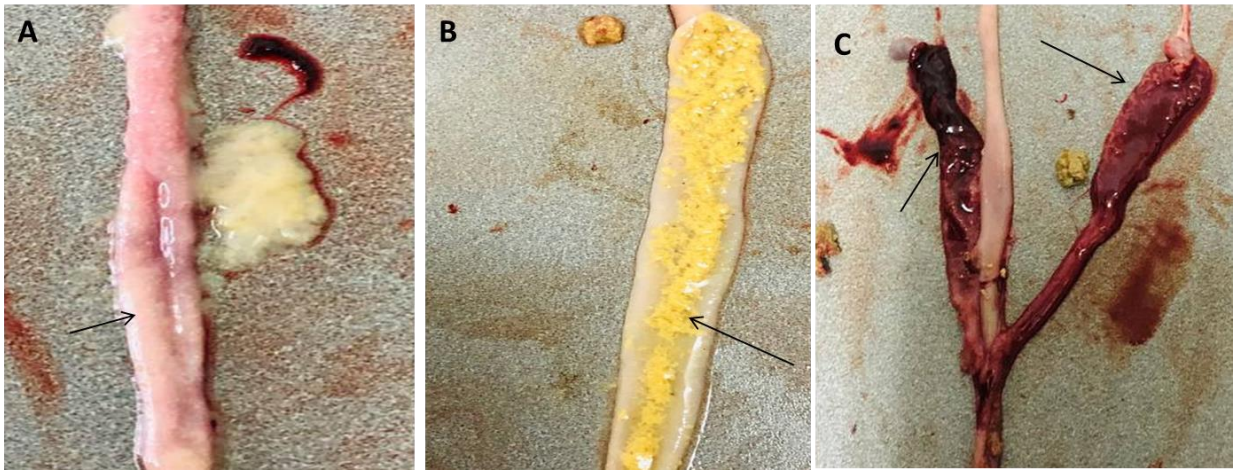


**Figure 7**: Pictorial presentation of the duodenum, jejunum and cecum

(A), Duodenum (B) Jejunum and Cecum (C) showing advanced lesions at score 3 or 4 characterized by thickened duodenal wall, coalescent lesion (arrow) and mucus accumulation, Jejunal wall thickening with watery content (arrow) and highly hemorrhagic cecum with little cecal content.

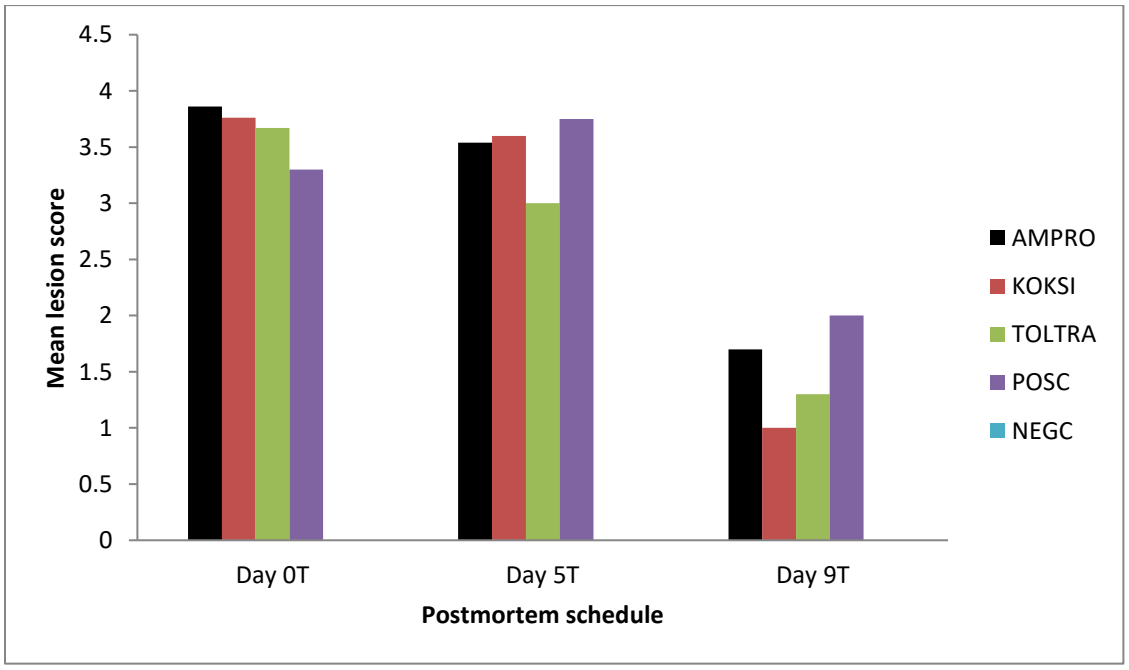


**Figure 8:** Mean lesion scores at different time points in infected chicken with or without treatment

### 4.4.Overall Judgment on the Efficacy of the Three Anticoccidial Drugs

The four indices estimated to determine anticoccidial drug efficacy were anticoccidial index (ACI), Relative Oocysts Production (ROP), Relative Lesion Score (RLS), and Percent Optimum Anticoccidial Activity (POAA). Based on the calculated values, strong resistance was detected against Amprolium and Toltrazuril since they did not they did not demonstrate minimum values for efficacy. On the other hand, moderate resistance was detected against Koksidex since it has failed in two of the four efficacy indices (Table 5).

**Table 5**: Evaluation of anticoccidial drug efficacy based on efficacy assessment indices

| Group | BW-0PT | BW-9PT | GSR | % weight change | survival rate | OPG | RRBWG | ROP | mean Lesion score | RLS | | ACI | | ROP | | POAA | | overall |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | value | efficacy | value | efficacy | value | efficacy | value | efficacy | |
| AMPRO | 262 | 411 | 1.57 | 0.57 | 69 | 86700 | 1.1 | 43.7 | 1.7 | 15 | R | 24.6 | R | 43.7 | R | 18.4 | R | R |
| KOKSI | 268 | 420 | 1.57 | 0.57 | 78.6 | 598 | 1.1 | 0.3 | 1 | 50 | S | 78.4 | R | 0.3 | S | 17.4 | R | R |
| TOLTRA | 275 | 418 | 1.52 | 0.52 | 92.9 | 179500 | 1.0 | 90.5 | 1.3 | 35 | R | 2.0 | R | 90.5 | R | -14.3 | R | R |
| POSC | 266 | 410 | 1.54 | 0.54 | 79.3 | 198300 | 0.78 | | 2 | | | | | | | | | |
| NEGC | 290 | 490 | 1.69 | 0.69 | 100 | 0 | 1.28 | | | | | | | | | | | |

OPG: Oocyst per gram, GSR: Growth and survival rate, BW: Body weight, RRBWG: Rate of relative body weight gain, R: Resistant, S: Sensitive, RLS: Reduction of Lesion score, ROP: Relative Oocyst Production

# 5. DISCUSSION

Poultry coccidiosis is one of the most important parasitic diseases that affect health and productivity of chickens and cases huge economic impact on the sector (Allen and Fetterer, 2002). Specifically, the *Eimeria* species exclusively target different sections of the intestine (Saeed and Alkheraije, 2023). The use of anticoccidial drugs like ionophores and chemicals for treatment of poultry coccidiosis is the main strategy to limit the impacts of the disease. However, the emergence of anticoccidial resistance by *Eimeria* species has become a growing concern worldwide (Abad and Ghaniei, 2023). This research has evaluated both in vitro and in vivo, the effectiveness of anticocidial drugs commercially available on the local market. The preliminary survey revealed that Amprolium (AMPRO), Koksidex (KOKSI) and Toltrazuril (TOLTRA) were the most preferred and sold anticoccidials in Bishoftu town. A study by Arowolo *et al*. (2012) similarly reported that Amprolium, together with other chemicals, was the preferred choice among the majority of poultry farmers.

## 5.1.Effect of anticoccidials on oocyst sporulation *in vitro*

Under natural conditions, coccidia infected chicken treated with anticoccidial drugs may shed oocysts for several days after the treatment is started. To simulate the effect of exposure of unsporulated oocysts to such drugs (Mathis and McDougald, 1981) on subsequent sporulation potential, an *in vitro* experiment was performed. The observation that KOKSI reduced sporulation rate of oocysts may suggest that treatment of chicken with this chemical may reduce the potential of fecal oocysts to develop in the litter and re-infect existing batch or contaminate new ones. This outcome agrees with the findings of Adulugba *et al.* (2017), in which they reported synergistic effect of Amprolium+Sulfaquinoxline combination on control of coccdosis. The lower percentage of sporulation observed with the combination therapy could indicate that the addition of Sulfaquinoxaline potentiated the inhibitory effects on *Eimeria* development than

Amprolium alone. On the contrary, Murshed *et al*. (2023) and Desalegn and Ahmed (2020) have reported a much stronger inhibitory activity of AMPRO against sporulation potential of *Eimeria* oocysts *in vitro*. This could be due to differences in the methodology or intensity of exposure of parasites to the chemicals in the different study areas.

## 5.2.Efficacy of anticoccidial drugs against poultry coccidiosis in experimental chickens

Clinical coccidiosis is often accompanied by overt clinical manifestations especially in young chicken (Julie, 1999). In this study, the signs observed in experimentally infected chickens with mixed *Eimeria* species included depression, reduced feed intake, ruffled feather, huddling and diarrhea of bloody, mucoid or watery nature. This started on the 3rd day post-infection, which is consistent with the typical incubation period of the disease (Amer *et al.,* 2010*;* Elmusharaf *et al.,* 2010*).* Bloody diarrhea was prominent on $5^{th}$ -$6^{th}$ day post infection which is consistent with the description by El-Banna *et al*. (2005). The decrease in clinical signs in infected groups including the positive control in subsequent days suggests a natural progression of the disease which may be mediated by the chickens' immune system (Lee *et al*., 2022).

Body weight measurement is one of the parameters often evaluated to assess the impacts of a disease or the response to treatment (Ojimelukwe *et al*., 2018). This study revealed that treatment with any of the three anticoccidial drugs did not significantly improve body weight gain in infected chicken. The only difference observed was that the non-infected non-treated group (NEGC) had significantly higher body weight on the $5^{th}$ and $9^{th}$ day post treatment compared to the infected groups irrespective of treatment status. This finding is in agreement with the observations of El-Ghany *et al* (2007) and Amer *et al* (2010) for AMPRO and KOKSI and Ahmed*et al.* (2023) for TOLTRA. This may suggest that treating animals at advanced stage of the disease might improve clinical signs but this may not be translated into body weight change in short time. It needs further study to know if this situation changes over long observation time. It is also possible that, the drugs were note effective enough to bring about body weight

improvements in treated groups. On the contrary, Adulugba *et al.* (2017) reported positive effect of AMPRO and KOKSI Jamilu *et al.* (2022) on body weight gain of experimentally infected chickens.

If anticoccidial treatments are effective, this expected to translate into significant fall in fecal oocyst count compared to the positive control group. The fact that oocysts per gram of feces (OPG) drastically declined on the 7$^{th}$ day of the infection (day 2PT) suggests that this is a natural process which may not be ascribed to any treatment. However, it was clearly evident that treatment has significantly contributed to the reduction in OPG count on the following days compared to the POSC group; the effect with KOKSI being more prominent. It appears that KOKSI was the best relative to TOLTRA and AMPRO which supports the in vitro observation in this study with the reason already explained by Chapman and Rathinam (2022).

This study has also compared the survival rate of the experimental groups. Based on the result, TOLTRA significantly reduced mortality compared to other anticoccidial drugs. This is in line with the report of Allam *et al*. (2008). In support of this observation, another study has also concluded that the mortality rates for TOLTRA treated experimental chicken was much lower compared to infected non-treated chickens (Alnassan *et al*., 2013).

Poultry coccidiosis often causes significant damage to the intestine resulting in observable gross lesions. The location and extent of lesions varies with the location along the length of the intestine and the species of the parasites; with preferential predilection sites (Conway, 1979). In this study, intestinal lesion scoring was conducted using the scoring technique described by Johnson and Reid (1970) at different time points. The observation of severe lesions (score 3 and 4) in the early days of infection, especially in the cecum, strongly agrees with the very high fecal oocyst count and prominent clinical signs recorded during this period. While the Duodenum, Jejunum and cecum were the common sites where gross lesions were detected, characteristically, the lesions appear to be of *E. acervulina* in the first two sections and of *E. tenella* in the cecum. Following

treatment, however, lesion scores declined but this also happened in the non-treated group implying that the improvement in gross pathology may be due to the natural progression of the disease rather than the effect of treatment. Ojimelukwe *et al.* (2018) has also demonstrated in experimental chickens that lesions in the cecal region were present in all infected groups regardless of treatment. However, this report has also revealed that overall lesion scores were less in birds of the medicated groups compared with the infected-unmedicated group. Similarly, Banna *et al.* (2005) and Chapman and Rathinam (2022) in their studies have shown that TOLTRA and KOKSI were effective in reducing lesion scores compared to the untreated control. Although, the observation time was short and the number of chicken euthanized for lesion scoring was small, the finding in the current study may suggest that all the three drugs tested were not effective in terms of improving intestinal pathology.

### 5.3.Overall efficacy of Amprolium, koksidex and Toltrazuril

For evaluation of anticoccidial efficacy tests in chickens, slightly differing assessment indices are used (Lan *et al.,* 2017, Ojimelukwe *et al*., 2018). This study employed the parameters used by Lan *et al* (2017) where anticoccidial index (ACI), Percentage of optimum anticoccidial activity (POAA), reduction of lesion score (RLS) and reduction of oocyst production (ROP). Accordingly, the findings unequivocally showed development of strong resistance to Amprolium and toltrazuril and moderate resistance to Koksidex. This is not surprising because, both the in vitro oocyst sporulation study and OPG count in treated animals that KOKSI was better than the other two drugs. Lan *et al.* (2017) have also reported different degrees of resistance to different anticoccidial drugs. Ojimelukwe *et al*. (2018) detected resistance in populations of *E. tenella* against toltrazuril. This result also agrees with the findings of Mesa *et al.* (2021).

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## 6. CONCLUSION AND RECOMMENDATIONS

This study was initiated with the objective of assessing the efficacy of anticoccidial drugs commonly used in commercial poultry farms in and around Bishoftu. It was observed that Amprolium, Koksidex and Toltrazuril are curative drugs widely abailable in local veterinary drug shops and frequently demanded by farms in bishoftu. However, this study has unequivocaly demonstrated that strong resistance has already developed against Amprolium and Toltrazuril while moderate resistance was detected for Koksidex. Although, from the observation of intestinal lesions it was evident that *E. acervulina* and *E. tenella* are with high probability on the basis of lesion localization, this study however has not differentiated which species of *Eimeria* has developed resistance.

Based on the above conclusion, the following practical recommendations are forwarded:

- Farms are advised to pose the use of Amprolium and Toltrazuril for some time so that susceptible populations emerge overtime
- Farms should apply Koksidex based on the severity of clinical cases in a flock, rather than using it indiscriminately.
- Farm extension programs should be strengthened to enable implementation of enhanced biosecurity protocols and reduce dependence on drug treatment
- It is recommended to consider the use of herbals as potential alternative methods for coccidiosis control.
- Further study is required to differentiate which *Eimeria* species are most resistant and to investigate alternatives such as novel anticoccidials and vaccines

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## 8. APPENDICES

**Appendix I:** Questionnaire survey

**Addis Ababa University College of veterinary medicine and agriculture**

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### I. Questionnaire survey collection format for FARM OWNERS

Thank you for giving your time to complete this questionnaire. This questionnaire survey is intended to gather information regarding anticoccidial drug use in and around Bishoftu. Please answer the following questions to the best of your knowledge. All responses provided in the questionnaire will be kept confidential and used solely for research purposes. Participants' personal information will not be disclosed or shared with any third party.

**So are you willing to participate in this study?_____ if yes, continue. If no, Stop**

**Date ...../...../..... Questionnaire No. .....**

**Section 1: General Information**

1.1.Farm Identification:_____

1.2. Farm Location (Address)_____

1.3. Farm size (number of birds):_____

1.4.Type of chickens (e.g., broilers, layers, breeders):_____

**Section 2**: **Coccidiosis**

2.1. Have you encountered problem of coccidiosis in your chickens?

**A**) Yes **B**) No **C**) Do not know

2.2. If your answer is yes, which of the following signs do you remember?

**A)** Watery, mucoid or bloody diarrhea

**B)** Decreased egg production in adult chickens

**C)** Poor growth

**D)** Droopy posture and wings with ruffled feathers

**E)** Many young chicken affected at one time

**F)** Mortality

2.3. If yes to the above question, how did you manage the problem?_____

**Section 4: Anticoccidial drug usage as treatment**

4.1. At times of coccidiosis problem, have you administered anticoccidial treatment?

**A)** Yes **B)** No

4.2. If your answer is yes to the above question, mention the name or type of drug used_____

## II. Questionnaire survey collection format for POULTRY PHARMACEUTICAL SUPPLIERS

**Date ...../...../..... Questionnaire No. .....**

**Section 1: General Information**

1.1.Name of establishment:_____

1.2.Location (Address)_____

1.3.Type (feed manufacturer/feed distributor):_____

**Section 2: Coccidiostat**

2.1.Do you sell coccidiostats for prophylactic purposes? **A)** yes **B)** No

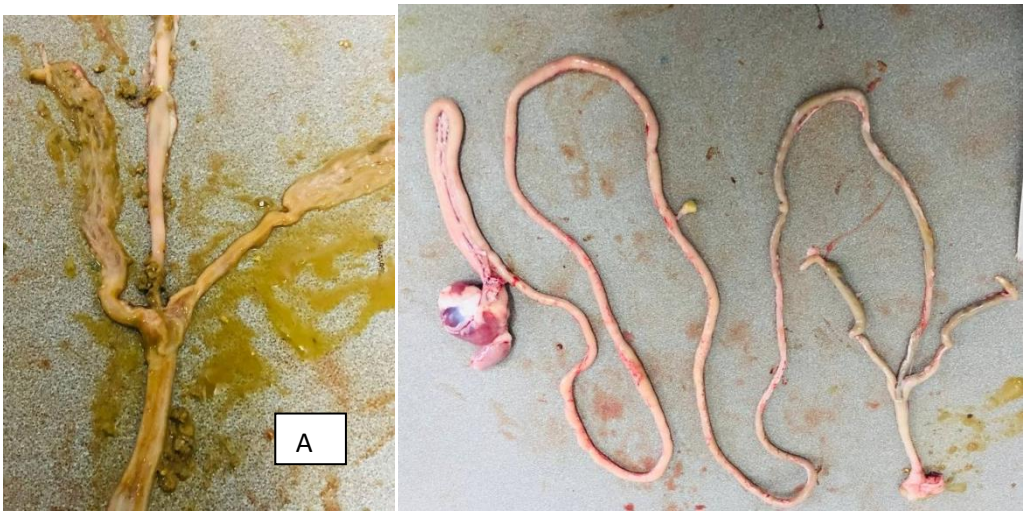
2.2. If yes, which one is most preferred by customers? _____

2.3.Do you sell anticoccidial drugs for treatment of infections? **A)** yes **B)** No

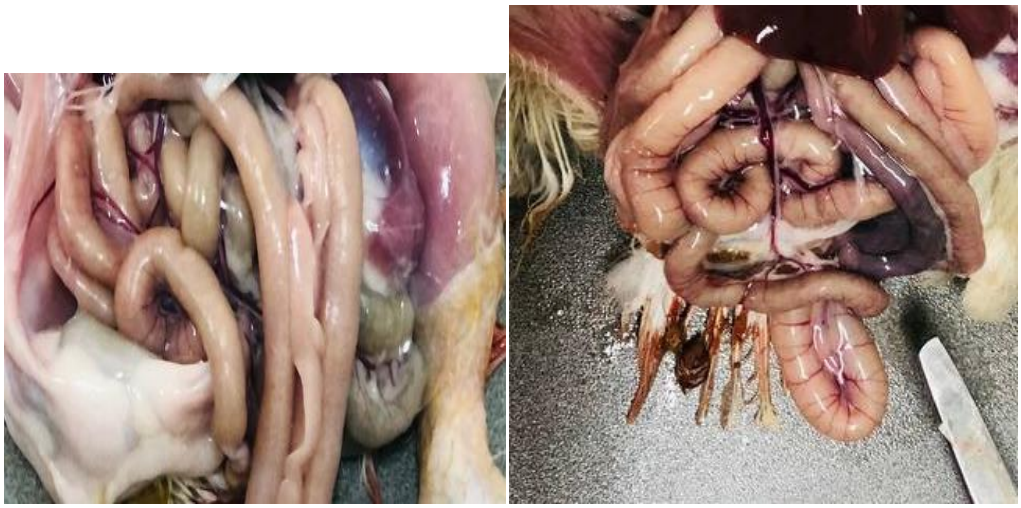
2.4.If yes, which one is most preferred by customers? _____

2.5.Can you please provide leaflets for each product or any information about recommended dosage or dilution?

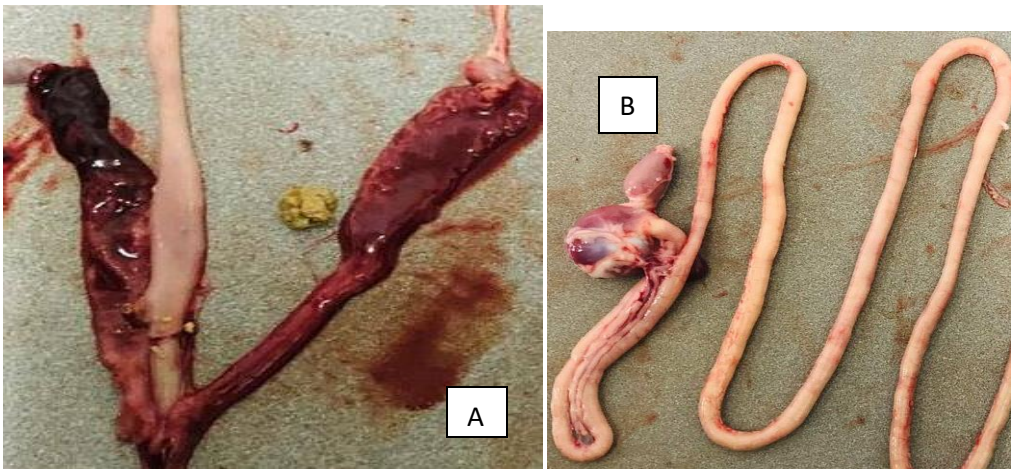
**Appendix II:** Post mortem examinations




Cecum and ileum of negative control GIT of negative control

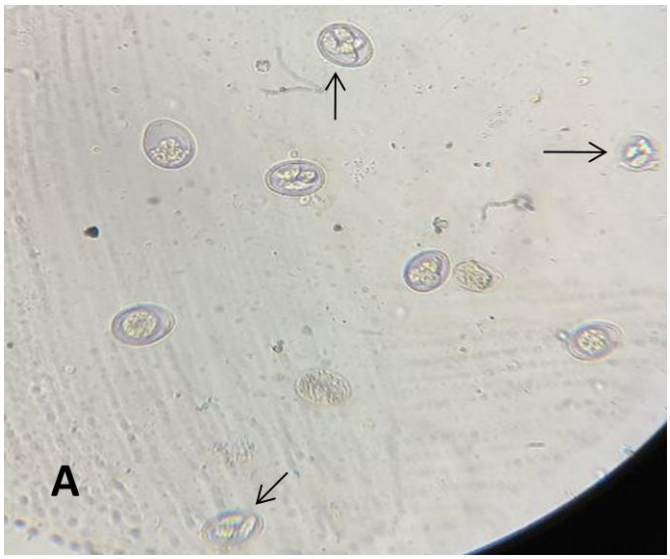


A, intact intestine of chicken from negative group B, Inflamed intact intestine

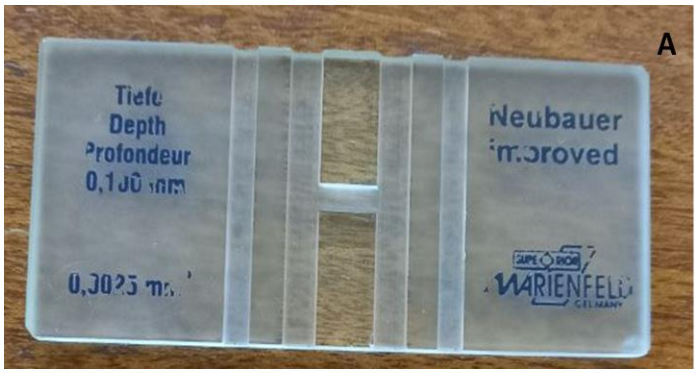



A) Hemorrhagic cecum and B) Distended duodenum and jejunum

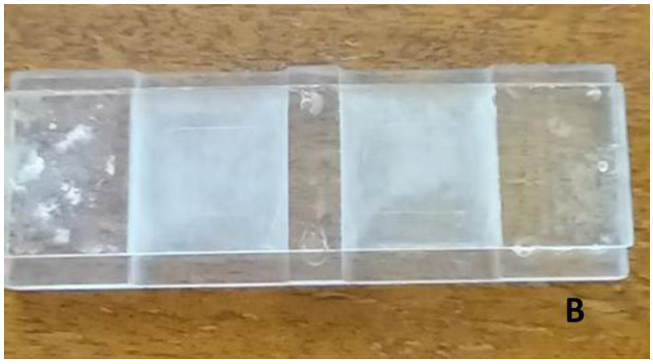
**Appendix III:** Laboratory and field pictures




**A),** arrows indicate sporulated *Eimeria* oocyst



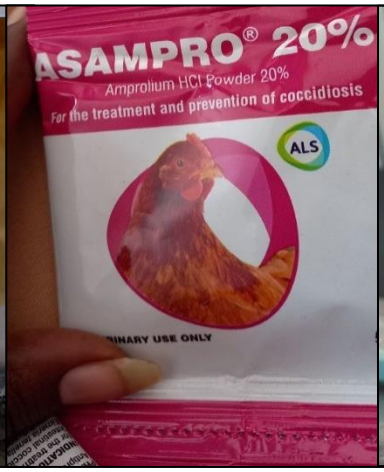

A) Neubauerhemo cytometer
B) MacMaster chamber

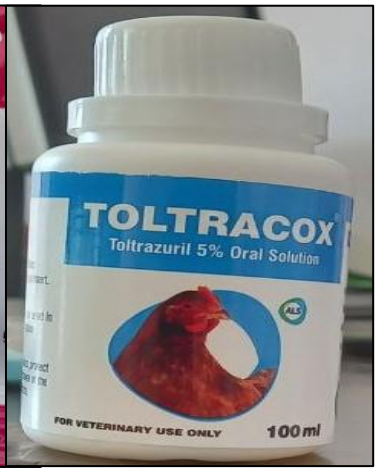



In vitro testing (left)

Fecal sample collection from farms (right)

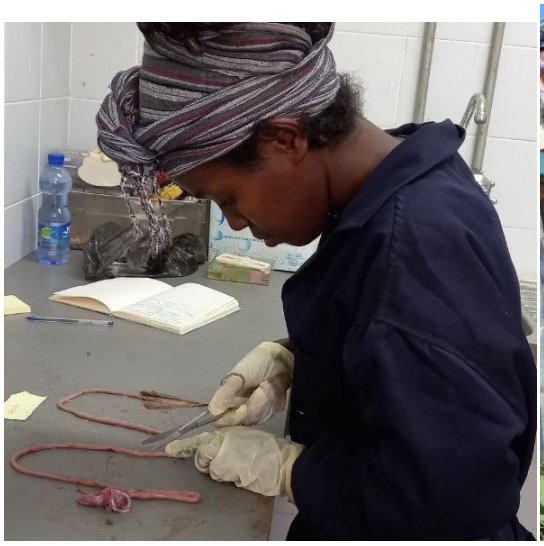

Taking humidity and temperature of the chickens

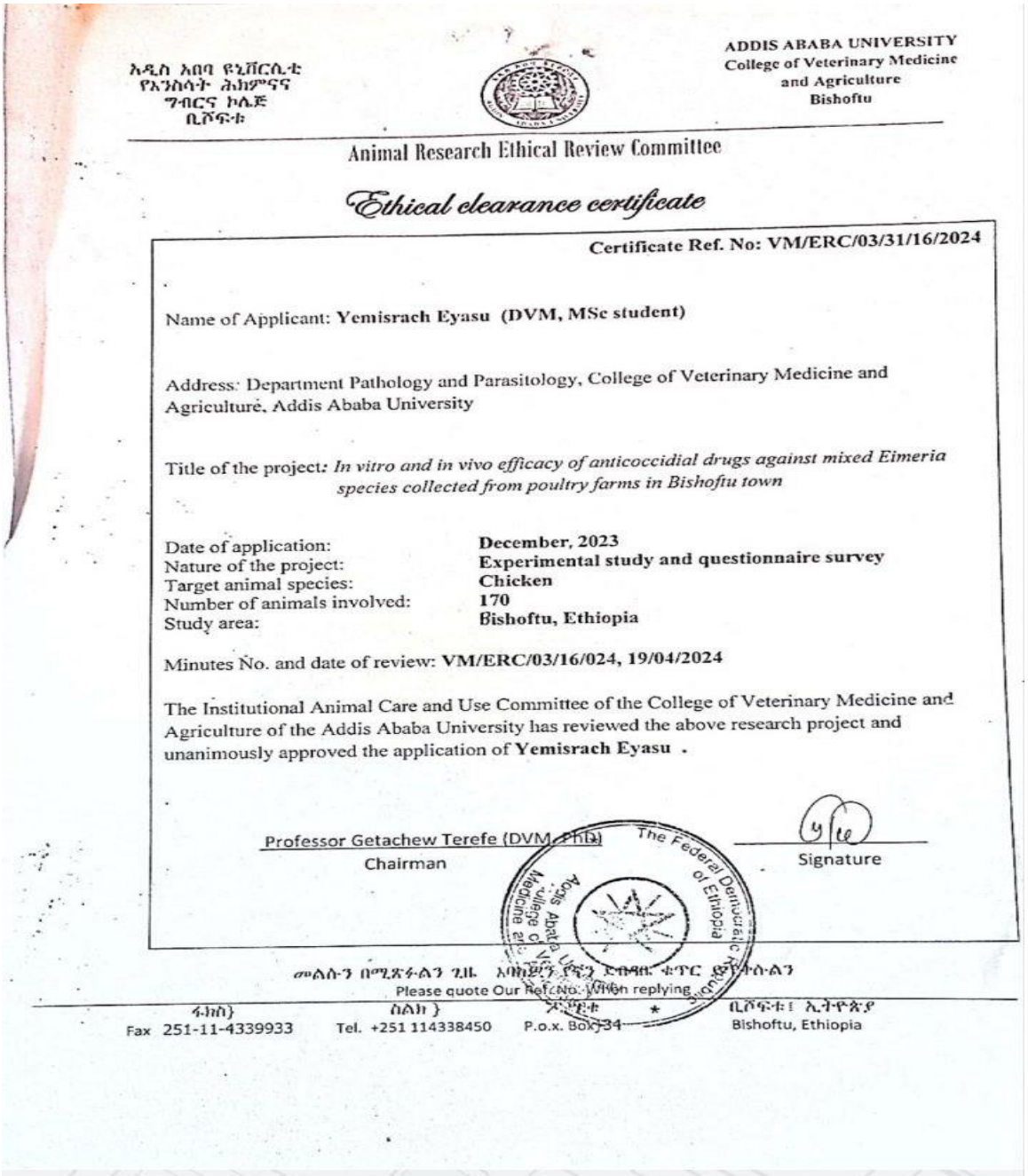

**Koksi** **Ashampro** **Toltracox**


Chickens experimental house Body weight measuring



Post mortem examination Conducting questionnaire

**Appendix IV**: Ethical Clearance



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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/03/31/16/2024**

Name of Applicant: **Yemisrach Eyasu (DVM, MSc student)**

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

Title of the project: *In vitro and in vivo efficacy of anticoccidial drugs against mixed Eimeria species collected from poultry farms in Bishoftu town*

| | |
|---|---|
| Date of application: | **December, 2023** |
| Nature of the project: | **Experimental study and questionnaire survey** |
| Target animal species: | **Chicken** |
| Number of animals involved: | **170** |
| Study area: | **Bishoftu, Ethiopia** |

Minutes No. and date of review: **VM/ERC/03/16/024, 19/04/2024**

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 **Yemisrach Eyasu** .

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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Please quote Our Ref. No. When replying

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