MORPHOLOGICAL AND MOLECULAR IDENTIFICATION OF *EIMERIA SPP*. OF BROILER CHICKENS IN NAGPUR REGION (MAHARASHTRA STATE)

T H E S I S

Submitted

In partial fulfillment of the requirements for the Degree of

M A S T E R O F V E T E R I N A R Y S C I E N C E
IN
VETERINARY PARASITOLOGY

BY
DESHMUKH PRANAV PRALHADRAO
Enrollment No: V/15/060

Nagpur Veterinary College, Nagpur

MAHARASHTRA ANIMAL AND FISHERY SCIENCES UNIVERSITY, NAGPUR - 440001
(INDIA)

2023

DECLARATION OF STUDENT

I hereby declare that the experimental research work and interpretation of the thesis entitled, **"MORPHOLOGICAL AND MOLECULAR IDENTIFICATION OF *EIMERIA SPP.* OF BROILER CHICKENS IN NAGPUR REGION (MAHARASHTRA STATE)"** or part thereof has not been submitted for any other degree or diploma of any University, nor the data have been derived from any thesis/publication of any University or scientific organization. The sources of materials used and all assistance received during the course of investigation have been duly acknowledged.

Date:

Place: Nagpur

Signature

(DESHMUKH PRANAV PRALHADRAO)
Enrollment No: V/15/060

(Dr. S. G. Jadhao)
Counter signed by
Chairman,
Advisory Committee with date

DECLARATION OF ADVISORY COMMITTEE

Mr. Deshmukh Pranav Pralhadrao has satisfactorily prosecuted his course of research for a period of not less than two semester and that the thesis entitled **"MORPHOLOGICAL AND MOLECULAR IDENTIFICATION OF *EIMERIA SPP.* OF BROILER CHICKENS IN NAGPUR REGION (MAHARASHTRA STATE)"**submitted by him is the result of research work is sufficient to warrant its presentation to the examination in the subject of **VETERINARY PARASITOLOGY,** for the award of **MASTER OF VETERINARY SCIENCE** degree by the Maharashtra Animal and Fishery Sciences University, Nagpur.

We also certify that the thesis or part thereof has not been previously submitted by him for a degree of any other University.

Place: Nagpur

Date:

(Dr. S. G. Jadhao)
Advisor/Guide
Assistant Professor
Department of Veterinary Parasitology
Nagpur Veterinary College, Nagpur

Name and Designation of Advisory Committee

	Name	Designation	Signature
1.	Dr. S. G. Jadhao (Chairman)	Assistant Professor Dept. of Veterinary Parasitology	______________
2.	Dr. S.W. Kolte (Member)	Professor& Head Dept. of Veterinary Parasitology	______________
3.	Dr. S.V. Shinde (Member)	Assistant Professor Dept. of Veterinary Public Health	______________
4.	Dr. M. M. Kadam (Member)	Associate Professor Dept. of Poultry Science	______________
5.	Dr. M. P. Kaore (Member)	Assistant Professor Veterinary Pathology	______________

CERTIFICATE

This is to certify that the thesis entitled, **"MORPHOLOGICAL AND MOLECULAR IDENTIFICATION OF *EIMERIA SPP.* OF BROILER CHICKENS IN NAGPUR REGION (MAHARASHTRA STATE)"**submitted by Mr. **Deshmukh Pranav Pralhadrao** to the Maharashtra Animal and Fishery Sciences University in partial fulfillment of the requirement for the degree of M.V.Sc has been approved by the Student's Advisory Committee after examination in collaboration with the External Examiner.

Name & Signature of
External Examiner

Signature with Seal
Head of Department

(Dr. S. G. Jadhao)
Advisor/Guide
Assistant Professor
Department of Veterinary Parasitology
Nagpur Veterinary College, Nagpur

Name and Designation of Advisory Committee

	Name	Designation	Signature
1.	Dr .S. G. Jadhao (Chairman)	Assistant Professor Dept. of Veterinary Parasitology	______________
2.	Dr. S.W. Kolte (Member)	Professor& Head Dept. of Veterinary Parasitology	______________
3.	Dr. S.V. Shinde (Member)	Assistant Professor Dept. of Veterinary Public Health	______________
4.	Dr. M. M. Kadam (Member)	Associate Professor Dept. of Poultry Science	______________
5.	Dr. M. P. Kaore (Member)	Assistant Professor Veterinary Pathology	______________

Associate Dean
Nagpur Veterinary College,
Nagpur

ACKNOWLEDGEMENTS

"जयाकारणे हे विश्व पाहतो मी, वर्धतो कणाकणाने अन सुखी नाहतो मी,
जयाकारणे निर्धास्त, अभिमानी हिंडतो मी, समस्तांस साभार हा प्राण मांडतो मी"

Acknowledging the good people that you already have in your life is the foundation for all abundance.

Better than a thousand days of study is one day with a great teacher. *Words can never express my gratitude for* ***Dr. Suresh Jadhao,*** *I never forget your support, guidance, and many things you did for me with your royal and dashing nature, thank you so much sir you are the greatest of all time mentor for academic and extracurricular activities.*

It was my luck, that I completed my master's under the guidance of great parasitologist, ***Dr. S. W. Kolte****, Professor and Head, Department of Veterinary Parasitology, NVC, Nagpur, Thank you, sir, for always supporting and believing in me.*

I acknowledge my sincere thanks to ***Dr. N.V. Kurkure****, Director of research (MAFSU) and Professor & Head Department of Veterinary Pathology, NVC, Nagpur, for allowing me to work under your valuable guidance.*

I also take the opportunity to express my sincere thanks to ***Dr. A. P. Somkuwar****, Associate Dean, NVC, Nagpur.*

I am grateful to all members of my advisory committee, ***Dr. M. M. Kadam****, Associate Professor & Head, Department of poultry science* ***Dr. S. V. Shinde****, Assistant Professor, Department of Veterinary Public Health,* ***Dr. M. P. Kaore****, Assistant Professor Department of Veterinary Pathology, for their constructive ideas during my research.*

My sincere thanks to ***Dr. Priya Gawande*** *&* ***Dr. Pratibha Jumde****, the teaching faculty of the Department of Veterinary Parasitology for their valuable guidance.*

I would like to thank very kind human being & helpful person ***Dr. A. P. Dhok****, Assistant Professor Department of Animal Nutrition &* ***Dr. Madhuri Hedau****, Assistant Professor Department of Veterinary Pathology,* ***Dr. Darshana Bhaisare*** *Assistant Professor, Department of poultry science for their constant help during this work. I am also thankful to Shri.* ***S. N. Gawande Sir*** *for providing me with necessary access to the library and for guiding me through this dissertation.*

I would love to express my gratitude towards my all family members, thank you for always believing in me. My dear brothers **Aniruddha & Prasad** thank you so much for your continuous support during this journey.

“EACH FRIEND REPRESENTS A WORLD IN US, TRUE FRIENDS MAKE GOOD TIMES BETTER AND HARD TIMES EASIER”

Dr. Sanket, you are the reason for my happiness, thank you so much for being there with me in every situation. **Dr. Omkar & Dr. Vaibhav,** I never felt that you guys are away from me, thank you for your support and encouraging me during my tough times. **Dr. Rushikesh, Dr. Vishal, Dr. Piyush, Dr. Roshnan, Dr. Kailas, Dr. Jayant, Dr. Pratik, Dr. Prashant, Dr. Piyush Bole, & Dr. Rajkumar,** thank you for making my life more cheerful.

I would like to thank **Dr. Sudhanwa Khardenavis,** for being such an amazing colleague. I am thankful to my all PG colleagues **Dr. Pratiksha, Dr. Pooja, Dr. Aniket, Dr. Naina, Dr. Gajanan, Dr. Ashwini, and Dr. Vrushali,** for their continuous help during my research work and for making these two years memorable.

My dear junior **Dr. Pratik** (Koki), this work is completed only because of you, thank you so much for your all-time helpful behavior. I am thankful to my junior **Dr. Hashal Bokde** for his much-needed help. My sincere thanks to my juniors, **Dr. Trupti & Dr. Pranita** for their help.

This acknowledgment is incomplete without your name **Sakshi**, thank you for your constant support, for improving me as a person, and for enhancing positivity in my personality.

I would love to give special thanks to **Swapnil bhau**,**Tahseen bhai, Sudesh kaka & Krushna bhau**, for their help during these two years.

There are too many people, who helped me during this journey, Thank You So much for being there with me.

Date: **Dr. Pranav Pralhadrao Deshmukh**

Place: Nagpur

TABLE OF CONTENTS

Sr. No.	CHAPTER	PAGE NO.
I	INTRODUCTION	1-4
II	REVIEW OF LITERATURE	5-23
III	MATERIALS AND METHODS	24-35
IV	RESULTS AND DISCUSSION	36-47
V	SUMMARY AND CONCLUSIONS	48-49
A)	BIBLIOGRAPHY	i-xx
B)	APPENDIX	xxi
C)	VITA	xxii
D)	THESIS ABSTRACT	xxiii-xxv
E)	प्रबंधसारांश	xxvi-xxviii

LIST OF TABLES

Table No.	TITLE	Page No.
3.1	List of primers used, its sequences and amplicon size	**30**
3.2	Cyclic conditions in Master gradient cycler for Universal primer, *Eimeria brunetti* and *Eimeria tenella*	**30**
3.3	Cyclic conditions in Master gradient cycler for *Eimeria acervulina* and *Eimeria mitis*	**31**
3.4	Cyclic conditions in Master gradient cycler for *Eimeria necatrix* and *Eimeria praecox*	**31**
3.5	Cyclic conditions in Master gradient cycler for *Eimeria maxima*	**31**
3.6	PCR reaction component for *Eimeria spp*.	**32**
4.1	Results of morphometric analysis	**37**
4.2	GenBank reference sequence & its country	**40**
4.3	Results of weekly OPG count	**43**
4.4	Results of intestinal gross lesion score	**43**

LIST OF FIGURES

Fig. No.	Name	After pages
4.1	Graph showing prevalence of coccidiosis	**37**
4.2	Graph showing comparative species prevalence	**37**
4.3	GenBank blast search for *Eimeria spp.* (Universal primer COI gene) haplotype.	**39**
4.4	GenBank blast search for *Eimeria acervulina* haplotype.	**42**
4.5	GenBank blast search for *Eimeria tenella* haplotype.	**42**
4.6	GenBank blast search for *Eimeria mitis* haplotype.	**42**
4.7	Phylogenetic distance tree of *Eimeria tenella*(COI gene)	**42**
4.8	Phylogenetic distance tree of *Eimeria acervulina*	**42**
4.9	Phylogenetic distance tree of *Eimeria tenella* (ITS-1 gene)	**42**
4.10	Phylogenetic distance tree of *Eimeria mitis*	**42**
4.11	Graph showing weekly OPG count in different groups	**44**
4.12	Graph showing comparative lesion scores in different groups	**44**

LIST OF PLATES

Plate No.	Name	After pages
4.1	Sporulated oocyst of *Eimeria mitis (400x)*	**37**
4.2	Sporulated oocyst of *Eimeria tenella (400x)*	**37**
4.3	Sporulated oocyst of *Eimeria acervulina(400x)*	**37**
4.4	Unsporulated oocysts of *Eimeria spp. (400x)*	**39**
4.5	Ruptured oocyst by using glass beads *(400x)*	**39**
4.6	Sample agarose gel electrophoresis of PCR products	**39**
4.7	Section of Duodenum group A showing numerous endogenous stages of *Eimeria* in enterocytes of villi and cellular infiltration H&E, 100x	**46**
4.8	Section of Duodenum group A showing lymphoid aggregates with shortening of villi and subserosal hemorrhages H&E, 100x	**46**
4.9	Section of Duodenum group B showing crypt necrosis and endogenous stages of *Eimeria* in enterocytes of villi (arrow) H&E, 100x	**46**
4.10	Section of Duodenum group C showing disintegrated villi with few endogenous stages of *Eimeria* in enterocytes, H&E, 100x	**46**
4.11	Section of Jejunum group A showing Numerous endogenous stages of *Eimeria* in enterocytes H&E, 100x	**46**
4.12	Section of Jejunum group B showing few endogenous stages of *Eimeria* in enterocytes H&E, 100x	**46**
4.13	Section of Jejunum group C showing few endogenous stages of *Eimeria* in enterocytes H&E, 100x	**46**
4.14	Section of Ileum group A showing numerous oocysts having centrally located nucleus and thick wall H&E, 100x	**46**
4.15	Section of Ileum group C showing few endogenous stages of *Eimeria* in enterocytes, H&E, 100x	**46**
4.16	Section of caecum group A showing developmental stages of *Eimeria sp*. in mucosa with disintegrated villi and hemorrhages in the muscularies layer,H&E, 100x	**46**

4.17	Section of caecum group B showing Absence of developmental stages of *Eimeria sp*. in mucosa, H&E, 100x	**47**
4.18	Section of Caecum group C showing few developmental stages of *Eimeria sp*. in mucosa, H&E, 100x	**47**

ABBREVIATIONS

% : Per- cent

μg : Microgram

μl : Microlitre

^{0}C : Degree Celsius

Dl : Decilitre

< : Less than

> : Greater than

BP : Base pairs

DNA : Deoxyribo Nucleic Acid

DW : Distilled water

EDTA : Ethylenediaminetetraacetic acid

Et al : Et alia (and others)

EtBr : Ethidium bromide

Fig : Figure

Gm : Gram

Hr : Hour

Viz. : Namely

TLC : Total leucocyte count

lit. : Litre

Mg : Milligram

Min : Minute

Ml : Millilitre

Mm : Millimolar

NVC : Nagpur Veterinary College

Sec	:	Seconds
µl	:	Microlitre
µm	:	Micrometer
PBS	:	Phosphate Buffer Solution
PCR	:	Polymerase Chain Reaction
RPM	:	Revolution per minute
TAE	:	Tris-glacial acetic acid –EDTA
Spp.	:	Species
UV	:	Ultra violet
V	:	Volts

INTRODUCTION

The poultry industry is the backbone of the livestock economy in India. Millions of people depend on the poultry sector for income and employment (Jadhav and Nikam 2014). As compared to other diseases in poultry, coccidiosis has been reported as a major threat to poultry farming (Lawal *et al.,* 2016).

Coccidiosis in poultry is an enteric and intracellular parasitic disease caused by different species of a protozoan parasite of the genus *Eimeria* (Phylum: Apicomplexa, Class: Sporozoea, Subclass: Coccidia, Order: Eucoccidia, Family: Eimeriidae). Coccidiosis is the most common and most important disease in the economic aspect of poultry worldwide (Shirley *et al.,* 2005). It is one of the major diseases of poultry that play a static role in the growth of the poultry industry and broiler industry globally. It causes an economic loss of more than 3 billion US dollars (Dalloul and Lillehoj, 2006). In India, it has been estimated that the commercial broiler industry was a major sufferer of coccidiosis, where nearly 95.61 percent of the total economic loss occurred due to the disease (Bera *et al.,* 2010).

Infection due to *Eimeria* species is influenced by poor housing and bad management system of poultry and affects chickens of all ages. Due to an underdeveloped immune system, the infection begins at a younger age (Musa *et al.,* 2010) and is responsible for severe infection in the intestinal epithelium of poultry. *Eimeria* species are very host-specific. The eight species responsible for coccidiosis in chickens are *E. acervulina, E. brunetti, E. hagani, E. maxima, E. mitis, E. necatrix, E .praecox, and E. tenella*. Among these, *E. acervulina, E. brunetti, E. maxima, E. necatrix*, and *E. tenella* are highly pathogenic, which cause great economic loss to the poultry industry worldwide (Kawahara, *et al.,* 2008). Differentiating between different *Eimeria* species is quite challenging due to similar clinical findings and developmental characteristics, (Long and Joyner 1984).

Primarily identification of *Eimeria* species, carried out in the laboratory, has been conducted traditionally based on morphologic and pathologic aspects, like characteristics of oocyst, variations in the prepatent period, sporulation time, clinical signs, and intestinal lesions, and histopathologic characteristics (Joyner and Long 1974). Coccidiosis is host specific; it develops and multiplies intracellularly in the gut. The transmission of parasites in broilers is by the faeco-oral route via the ingestion of sporulated oocysts of *Eimeria spp.* (Dalloul *et al.,* 2005). Various factors affect the infectivity of sporulated oocysts, which includes the number of oocysts present in the litter material, chick density, susceptibility of birds, and immunogenicity (Chapman *et al.,* 2002).

Ingestion of sporulated oocysts via feed or water intake is responsible for the occurrence of infection. After the excystation of sporulated oocyst in the gut, infective form sporozoites are released. These sporozoites invade the epithelial linings of the intestine and cause intense tissue damage, which is responsible for causing secondary bacterial infections (Daszak, 1999). The clinical form of coccidiosis is characterized by loss of appetite, pale combs and wattles, ruffled feathers, blood-tinged feces, diarrhea, dehydration, and death, (Fanatico, 2006).

For the application of effective control strategies, the identification of *Eimeria* species is essential. The species involved in infection is often determined using the site of infection by observing gross lesions and using the intestinal lesion scoring technique (Conway and McKenzie, 2007).

For morphometric identification, *Eimeria* species were categorized according to the size of oocysts into three groups: small oocysts, <18.8µm tentatively *E. acervulina* and *E. mitis,* medium-sized oocysts 18.9µm to 23.8µm tentatively *E. necatrix, E. tenella*, and *E. praecox*, large oocysts, >23.9µm, tentatively *E. maxima* and *E. brunetti* (Haug, *et al.,* 2008). By simply using oocyst morphology, we cannot precisely distinguish the different species of *Eimeria*, as morphological features are similar for nearly all *Eimeria spp*. They have an ellipsoidal, ovoid, or circular shape and have thick cell walls, (Clark and Blake 2012). Mixed infections due to *Eimeria* species are pretty tricky for the precise discrimination of species using morphological methods. Alternatives for species-

specific diagnosis are required to inform routine animal husbandry, veterinary intervention, and epidemiological investigation, (Kumar *et al.,* 2014). Newly developed molecular techniques have allowed accurate diagnosis of *Eimeria* species, investigation of the genetic variability of these pathogens, and also helps in the molecular characteristics associated with phenotypical characteristics that may constitute the use of molecular markers (Schnitzler *et al.,* 1998; Costa *et al.,* 2001).

The accurate diagnosis of coccidiosis in poultry is primary for its effective control, and it is vital for the investigation of a disease outbreak of coccidiosis and for ensuring the effectiveness of the application of a live vaccine (Morris and Gasser, 2006). Traditional methods are not sufficiently reliable to allow a species-specific diagnosis of coccidiosis. Limitations of the traditional methods have been overcome by the development of various biochemical and molecular methods.

Detection and diagnosis of *Eimeria* species infections are mostly made through microscopic examination of the faecal sample and molecular and serological techniques. Faecal sample examination based on the presence of oocyst is the most straight forward and accessible diagnostic test for most veterinarians, but it is unreliable for differentiation between species. Molecular methods like PCR gives species level identification, which is further essential for developing vaccines.

Along with these techniques, we should remember that the early diagnosis of coccidiosis is still possible with the lesion-scoring technique. For an accurate diagnosis, proper selection of birds, standard necropsy procedure, and exact identification of lesions are essential. Control of coccidiosis is a major issue due to increasing resistance to synthetic anticoccidials. Alternative to this, we can use plant bioactive compounds that could be used as potent anticoccidial agents. Nowadays, due to increasing resistance to anticoccidial drugs and their residual effects, consumers request poultry products free from drug residues (Harper and Makatouni 2002). Using natural products or plant bioactive molecules as an alternative to these anticoccidials may be the best solution to this demand. As essential oils have antimicrobial activity, they can be used as anticoccidials. The

effectiveness of essential oils of thyme, clove, or tea tree oil at low concentrations could have significant benefits when used to treat coccidiosis along with good palatability, cost-effectiveness, and less toxic and residual effects (Remmal *et al.,* 2011).

The main objective of this work is to find out species of *Eimeria* present in the Nagpur region by morphometry and conventional PCR method and to check the anticoccidial effect of essential oil of Thymol on a coccidiosis-challenged group of broilers. The focus of this work is to enhance the study of the epidemiology of coccidial infections in commercial broiler flocks by studying the distribution of coccidial infections, prevalence, and the types of different *Eimeria* species commonly present in the Nagpur region.

OBJECTIVES:

1. Morphological identification of different *Eimeria* species in broilers droppings.
2. Molecular identification and characterization of different *Eimeria* species in broilers droppings.
3. To study the efficacy of essential oil of Thymol as anticoccidial agent in broilers.

REVIEW OF LITERATURE

2.1 History of Coccidiosis:

Coccidiosis is a disease caused by protozoan parasites of the genus *Eimeria* which belongs to the phylum Apicomplexa. Coccidian parasites infect vertebrates with a very wide host range and they are obligate intracellular parasites present in the intestinal tract of the vertebrate hosts and are very host specific. The population density of animals or birds is a predisposing factor for the manifestation of clinical Coccidiosis (Barta, *et al*., 2001).

The *Eimeria* species have a complex life cycle and all stages affect the intestinal tract of different species of birds (Peek and Landman2011). Sufficient Infection by *Eimeria* oocysts to produce clinical manifestations is termed clinical coccidiosis where as a mild infection that does not show clinical signs are known as coccidiasis (Conway and Mckenzie 2007). However, this terminology has been revised by WAAVP and it has been turned to coccidiosis even if a mild infection is detected. It is one of the major diseases of poultry that play a static role in the growth of the poultry industry and broiler industry globally. It causes economic loss of more than 3 billion US dollars (Dalloul and Lillehoj 2006). In India, it has been estimated that the commercial broiler industry was a major sufferer of coccidiosis, where nearly 95.61 percent of the total economic loss occurred due to the disease (Bera *et al.,* 2010).

Coccidiosis is the economically most important disease of broilers. Naturally, mixed infection of different *Eimeria* species is commonly observed in birds (Kumar *et al*., 2015). In domestic birds or broilers out of seven recognized *Eimeria* species *E. maxima, E. necatrix, E. brunetti,* and *E. tenella*, show very high and severe pathogenicity, *E. acervulina* and *E. mitis* show moderate to less pathogenicity, whereas *E. praecox* shows very less pathogenicity(Thebo *et al*.,1998).

Different species of *Eimeria* are confined to a specific location within the intestinal tract among the particular definitive host (Barta *et al.,* 1998).

Eimeria infection in birds is responsible for severe intestinal damage in the host which affects feed intake and increased mortality (Cha *et al*., 2014). As the parasite invades and multiplies in the cells of intestinal mucosa it damages the tissue which results in decreased feed intake and weight gain, increased feed conversion ratio, inflammation and necrosis of mucosal linings, malabsorption, diarrhoea followed by death (Dalloul and Lillehoj 2005; Williams 2002).

Each species of *Eimeria* causes a different form of disease like coccidiasis (mild form), subclinical coccidiosis, and clinical coccidiosis or frank disease (Williams, 2005). The negative effects of coccidiosis are also observed as birds become susceptible to necrotic enteritis due to secondary bacterial infection by *Clostridium spp*., (Persia *et al*., 2006).

2.2 Life cycle:

Transmission of intestinal coccidiosis is via the faeco-oral route. Coccidia follow an apicomplexan life cycle including both asexual and sexual phases of reproduction. There are four sporocysts present in each sporulated oocyst of *Eimeria* and each sporocyst contains two sporozoites which act as an infective agent within the intestine. The sporulated oocyst is an infective stage and can remain viable in the environment for months to years, under favorable conditions until its transmission (Barta *et al.,* 2001).

After ingestion of sporulated oocyst infection occurs. In the digestive tract of the chicken, sporozoites are released by mechanical action and chemical factors mainly trypsin, CO2, and bile (Conway and Mckenzie, 2007; Jones *et al.,* 1996); Allen and Fetterer, 2002). Within the host cell sporozoites penetrated and they transformed into trophozoites which undergo the asexual phase of reproduction in which nuclear division and cytoplasmic differentiation occur and merozoites (first-generation schizonts) are released in the gut wall, the process is known as schizogony or merogony. The released merozoites again penetrate new host cells and undergo second schizogony and release second-generation schizonts, (Allen and Fetterer, 2002; Innes and Vermeulen, 2006). There are 3-4 cycles of asexual reproduction within enteric cells occurs (McDonald and Shirley 2009).

The maturation of these merozoites occurs and gametes i.e. macrogametes & microgametes are formed. The gamets participate in the sexual phase of the life cycle and oocysts are produced. These oocysts are shaded into the feces (Long and Hotron-smith 1968). These released oocysts sporulate and become infective in the presence of moisture, oxygen, and warmth (Norton and Chard 1983). The total life cycle is of 4-5 days with slight species variation (Brown *et al.,* 2002).

2.3 Host specificity:

In poultry, most coccidias are classified within the genus *Eimeria*, which is known for its high host specificity. *Eimeria* species are known to exhibit a marked degree of host specificity which infects single species of host or a specific genus of a host (Becker, 1948; Long and Millard, 1979; Vbra and Pakandal, 2015).

Eimeria species which infect a wide range of host genera are very rare, (Vbra and Pakandal 2015). For example, species that infect the chicken are not infective to turkeys and vice versa. Several experiments were conducted to test for the cross-infectivity of Eimeria species in both these hosts.

Tyzzer (1929b), was unable to infect chickens with *E. meleagridis* from turkeys and turkeys with *E. acervulina* from chickens. Patterson (1933), failed to infect turkeys with *E. tenella* from chickens. Several unsuccessful attempts have been made to infect chickens, pheasants, guinea fowls, and Japanese quail with *E. adenoeides* of turkeys (Moore and Brown 1951).

2.4 Pathology and gross lesions:

For the development of the pathological condition, second-generation schizonts play a significant role. Host and species variation, nutritional status, associated diseases, and *Eimeria* species influence the pathological occurrence of coccidiosis (Nour 2020). As coccidiosis is a gut-associated disease and damage to the gut wall allows the development of secondary infections (Shirley and Harvey 2000).

Yellowish-colored diarrhoea is the most commonly observed symptom initially. With an increase in infection, the disease progresses and leads to blood loss in droppings due to intestinal hemorrhages, feces that are red or chocolaty in color, rapid loss in body weight, and increased thirst. The clinical manifestation of the disease starts when second-generation schizonts start to replicate rapidly (Soulsby, 1982).

On necropsy examination, the damaged intestinal wall shows thickening which resembles sausage (Nour, 2020). Lesion scoring technique with numerical ranks ranging from 0 to +4, has been commonly used to compare the gross lesions in coccidiosis-affected birds, it gives different grades of pathogenicity to the lesions. Species responsible for pathogenicity may be identified by positive scoring of the affected part of the intestine (Johnson and Reid, 1970).

Caecal lesions are mostly developed by *E. tenella* but certain strains also responsible for rectal coccidiosis, along with pinpoint hemorrhages and ballooning is also observed. *E. mivati* or *E. acervulina* are responsible for lesions in duodenal part of intestine. Mid-intestinal lesions are mostly associated with *E. necatrix*. *E. maxima* can induce lesions in any part of the small intestine. In the rectum or lower intestine *E. brunetti* is commonly found (Johnson and Reid, 1970; Conway and Mckenzie, 2007; Kant *et al.,* 2013). The lesion score and weight gain are related and this relationship is depending on the responsible species of *Eimeria*. This lesion score provides the severity of infection (Conway *et al.,* 1990).

2.5 Diagnosis:

In poultry, correct diagnosis of highly economic diseases like coccidiosis is important. Traditionally coccidiosis is diagnosed on basis of history, clinical signs, and symptoms, by examining droppings or litter material and by observing the gross intestinal lesions (Singla and Gupta 2013). These traditional methods are highly time-consuming and skilful person is required for accurate diagnosis (Long & Joyner 1984; Carvalho *et al*., 2011; Shirley *et al.,* 2005).

Diagnosis of coccidiosis can be made on detection of oocysts in feces, by examining gross as well as microscopic lesions and by molecular methods. Identification of strain and species of *Eimeria* is an important tool for management, control, and epidemiologic studies of coccidiosis (Woods *et al.,* 2000). Biotechnological advancements like molecular methods of species detection are useful in the diagnosis and control of coccidiosis (Morris and Gasser, 2006).

2.6 Prevalence of Coccidiosis in India and abroad:

For the diagnosis of this disease, accurate identification of *Eimeria* spp. is important as it also helps in the management of subclinical infection, application of effective control strategies, and improves biological and epidemiological study (Lee *et al.,* 2010; Sun *et al.,* 2009). An incidence of coccidiosis in poultry in India, as well as foreign countries, has been widely reported.

Total of 16 poultry farms were screened by Pant *et al.* (2018), and out of them 9 suffered from different *Eimeria* species infection with an overall prevalence of 56.25%. Among these 9 farms, 6(60%) were broiler farms and 3 (50%) were layer flocks. The maximum prevalence of coccidiosis was recorded within the duration of Aug-Sep (50%) which is followed by June-July (33.3%) and Dec-Jan (16.6%) in the case of broilers, while for layers there was the equal prevalence of (33.3%) during June-July, Aug-Sep, and Oct-Nov.

From June 2006 to May 2008, Nikam *et al.* (2012) examined a total of 2524 samples, 734 of which were positive for coccidial infection, with a prevalence of about (29.08%).

An overall prevalence of 39.58% was recorded by microscopic examination of 720 faecal samples collected from poultry farms of Jammu by, Sharma *et al.* (2013), and five *Eimeria* species were identified viz., *E. tenella, E. necatrix, E. maxima, E. acervulina* and *E. mitis*. They observed that *E. tenella* was the predominant species in both organized and unorganized farms.

Studies on the prevalence of gastrointestinal parasites in chickens reared under backyard and intensive systems were carried out by Kumar *et al.* (2015) in Uttar Pradesh and Uttarakhand. They screened a total of 58 poultry farms and 81.03 % were positive for *Eimeria* spp.

From February 2010 to January 2011, Kala *et al.* (2013) conducted a survey on 556 poultry birds at Central Poultry Breeding Farm, Patna, and in local poultry farms of Patna, on group broilers and layers. They observed incidence of coccidiosis in the study area was found to be 16.54%, based on the number of birds examined and it was higher in broilers (21.38%) as compared to layers (11.27%), in young (22.81%) than in adult (9.3%), and in the rainy season (32.14%).

Rao *et al.* (2012) carried out a study to check the prevalence of *Eimeria* infection in various agro climatic zones of southern India. They collected 21 samples from broiler farms and found that 13 (61.9%) and of a total of 12 samples from layer farms, 6(50%) samples were positive for single or mixed infection of *Eimeria spp.* Based on their study they recorded an overall prevalence of 57.57% of coccidiosis, in both broiler and layer farms.

From June 2013 – January 2014, a total of 704 faecal samples from Gangapur and 699 samples from the Vaijapur region of Aurangabad, Maharashtra were examined for coccidial infections by Jadhav and Nikam (2014), out of which 254(36.07%) samples from Gangapur region and 251(35.90%) samples from Vaijapur region were positive respectively. A comparative study shows minor differences in prevalence.

The prevalence rate of coccidiosis in the broiler Chicken Farms in Western Iran recorded by, Gharekhani *et al,* (2014) was 31.8%. The highest rate of *E. acervulina* (75.7%) was followed by *E. tenalla* (54.3%), *E. necatrix* (28.6%), and *E. maxima* (20%).

Karaer *et al.* (2012) collected faecal samples from 1,108 broiler houses in six regions. After microscopic studies using the modified McMaster method they concluded that the presence of *Eimeria* spp. oocysts in fecal samples were about 12% of all broiler farms in Turkey. The age of the chickens varied from 1 to 50 days. Oocysts were found in 602 (54.3%) of these broiler houses.

In Western Ethiopia, Oljira *et al.* (2012) conducted a study to determine the prevalence of poultry coccidiosis and to relate it with different risk factors in and around Ambo town, western Ethiopia from October 2010 to April 2011. Flotation for qualitative and McMaster counting techniques for quantitative studies was used. Out of the total 384 chickens examined, 79 (20.57%) were positive for coccidian parasites.

A survey for the prevalence of coccidiosis and identification of species found in Vom, Plateau state, Nigeria, was conducted by Muazu *et al.* (2008). Out of 300 samples from nine different poultry farms investigated from April to June. They observed the prevalence of coccidial infection among adults (36.7%) was lesser than the younger birds (52.9%). The species of *Eimeria* identified by them were *E. tenella, E. maxima, E. necatrix*, and *E. acervulina*.

In Northern Jordan, Mohammad *et al.* (2002) examined 200 broiler farms and observed the overall prevalence of coccidiosis was 78%. They found that E. tenella was the most prevalent species (50%) of the farms had all six chicks infected and many of the farms and birds had shown multiple and mixed infections.

Iqbal and Begum (2018) conducted a study to assess the prevalence of coccidiosis in broilers by randomly screening a total of 1000 farms in Bangladesh from July 2007 to June 2008. In their study, the general prevalence of coccidiosis in broilers was found 36%. They recorded the highest prevalence of coccidiosis in the age group of 15-21 days old (52%) followed by the age group 8-14 days (30%) and in the age group 0-7 day’s prevalence was (2%).

In Sweden, Lunden *et al.* (2000) collected litter material and droppings of layers from 57 flocks on 26 farms. Coccidiosis was diagnosed in 11 flocks (19·3%) from 9 (31%) of the farms. According to his study, outbreaks occurred when the birds were 19 to 32 weeks old. *E. maxima* were identified in 6 and *E. tenella* in 3 of the outbreaks.

In five different cities in the Mazandaran province, of North Iran, Shirzad *et al.* (2011) conducted a study on 120 broiler farms. He randomly selected five chicks (3–8 weeks of age) on each farm, post-mortem, and parasitological examinations were performed and five *Eimeria spp*. were recognized i.e. *Eimeria tenella, Eimeria maxima, Eimeria acervulina, Eimeria brunetti*, and *Eimeria necatrix*. He concluded that the prevalence rate of subclinical coccidiosis among them is 75% (90 farms out of 120). *E. acervulina* was the most prevalent species (65.5%) followed by *E. maxima* (17.7%), *E. tenella* (15.5%), *E. brunetti* (10%), and *E. necatrix* (5.5%).

In Japan, during the year 2017-2018, Matsubayashi *et al.* (2020) based on oocyst morphology and PCR analyses carried out the prevalence of *Eimeria spp*. on breeding farms in Japan. They collected a total of 143 samples from 37 breeding farms in 21 prefectures in Japan. Based on their study they found oocysts of seven species at 34 of 37 breeding farms by PCR and identified the prevalence of *E. brunetti* at 51.5% of farms. The differences in the identification of *Eimeria* spp. between the morphology and PCR assay methods of oocysts were pronounced for *E. maxima* and *E. necatrix*. They confirmed that molecular tools were more suitable for accurately estimating the prevalence of *Eimeria spp.,* and their findings suggested that *E. brunetti* could be widespread in Japan.

Prakashbhau *et al.* (2017) collected 107- fecal samples from northern India and 133 fecal samples from southern India. With help of oocyst morphology and genus-specific PCR-based analysis, they confirmed that 79.4% (85/107) farms from northern and 76% (101/133) from southern India were found to be positive for any *Eimeria spp*... In north India, all *Eimeria* species were detected, while only *E. praecox* was undetected in south India. The most common species were *E. tenella* followed by *E. mitis*, in both regions. *Eimeria acervulina* and *E.*

necatrix were also common. For all species, the occurrence was lower in the south than the north, and co-infection with more than one species was more common in northern India.

2.7 Morphometric identification of different *Eimeria* oocysts:

The different characteristics of *Eimeria* oocysts are important for the differentiation of species. Traditionally *Eimeria spp*. identified by their morphological structure, host range, shape & size (Hill *et al.,* 2012) but manual methods of identification are time-consuming and quite difficult (Adams *et al.,* 2022).

According to Levine (1982), there are specific differences between the oocysts of a particular genus of coccidia. Within a single genus, the special and main characters of *Eimeria* oocysts may be used to differentiate the species. In the case of the fowl, there is considerable overlapping of dimensions. The shape of the oocyst tends to be constant throughout the range of dimensions while the shape index (length/breadth) is quoted as a further specific character (Long and Joyner 1984).

Average of measurements of at least 100 sporulated oocysts including length & width of oocyst length & width of sporocyst, length: width ratios of oocyst and sporocyst are best for morphometric identification (Duszynski *et al.,* 1997).

Noting characteristic features like the smoothness of the outer wall, number of layers, presence of conical projections, and approximate thickness are important to differentiate the *Eimeria spp.* (McAllister and Upton, 1989).

The presence of micropyle, its location, width & depth are unique to characterize genus & species (Parker and Duszynski, 1986). As studied by Becker (1959), oocysts were ellipsoid to broadly ovoid, colourless, and had a micropyle with a smooth exterior. The oocyst wall may consist of three layers, the outer layer i.e. exo, middle, and inner one is the endomembrane.

For different *Eimeria* spp. average oocyst size is *E. tenella* (22x19), *E. necatrix* (20.4x17.2), *E. maxima* (30.5x20.7), *E. acervulina* (18.3x14.6), *E. mitis* (16.2x16), *E. praecox* (21.3x17.1), *E. brunetti* (24.6x18.8), *E. hagani* (18.1x16.5), *E. mivati* (15.6x13.4), (Edger and Seibold, 1964).

Amer *et al.,* (2010) measured dimensions (length/width) and recorded oocyst shape index & the values were, *E. tenella* (1.14), *E. acervulina* (1.27,1.25), *E. necatrix* (1.20,1.19,1.22), *E. praecox* (1.23).

According to Tyzzer (1929a), the shape & size of oocysts were, *E. acervulina* (19.5x14.3) with a shape index of 1.25, *E. maxima* (31x23), *E. mitis* (17x15) with an average shape index 1.1.

2.8 Molecular identification of different *Eimeria* oocysts:

The molecular method uses PCR assay by amplifying specific genes in the DNA sequences of *the Eimeria* parasite. In recent years, polymerase chain reaction (PCR) has been developed to provide accurate and rapid identification of the known *Eimeria* species of chickens.

By using, the internal transcribed spacer 1 (ITS-1)-based nested PCR, Bhaskaran *et al.,* (2010) studied, intestinal samples of chicks from commercial poultry farms, in India, which are suspected of having *Eimeria* infections. They also sequenced the PCR-amplified ITS-1 regions from these samples. Out of 26 field samples analyzed, 19 showed the presence of multiple infections of *Eimeria* spp. They recorded the highest incidence of *Eimeria tenella* (80%) followed by *Eimeria mitis* (53%), *Eimeria acervulina* (42%), *Eimeria brunetti,* and *Eimeria maxima* (23%). In the samples analyzed, the incidence of *Eimeria necatrix* was found to be the lowest (15%) while none of the samples analyzed showed the presence of ITS-1 sequence from *Eimeria praecox.*

A total of 75 poultry farms were screened in three north Indian states viz. Uttar Pradesh, Uttarakhand and Haryana by Kumar *et al.,* (2015). On microscopic examination of the fecal sample, they revealed that 61 (81.3%) were positive for *Eimeria spp.* of faecal samples. ITS-1-based nested PCR confirmed the

prevalence of *E. acervulina* (79.2%), *E. brunetti* (12.5%), *E. maxima* (64.6%), *E. mitis* (89.6%), *E. necatrix*(64.6%), *E. praecox* (60.4%) and *E. tenella* (97.9%) in all screened farms. Mixed infection was found in the majority of farms. They observed the presence of all seven species of *Eimeria* in three farms only. The highest incidence of *E. tenella* (97.9%) was found while *E. brunetti* was detected in only 12.5% of farms.

Tissue samples from different parts of the intestine were collected from chickens showing signs and symptoms of coccidiosis & used for DNA extraction by Aarthi *et al.,* (2010) in Tamilnadu, India. They amplified the internal transcribed spacer (ITS) region of *the Eimeria* genome with genus-specific primers and speciation in a nested polymerase chain reaction (PCR) with species-specific primers. Out of 43 tissue samples examined, they recorded that 25 were positive in ITS PCR and all seven species were identified with a variable prevalence of each species.

In Romania Gyorke *et al.,* (2013) examined prevalence by using the PCR method and they found infection of *Eimeria* spp. in 21 (91%) out of 23 flocks, and in 11 (92%) out of 12 farms. They identified and confirmed four species of *Eimeria* i.e. *E. acervulina, E. tenella, E. maxima, and E. praecox*. After overall studies, they concluded that the most prevalent species was *E. acervulina* (21/23; 91%), followed by *E. tenella* (14/23; 61%). Infection with only *E. acervulina* was detected in six (26%) positive flocks which originated from large farms.

Luu *et al.,* (2013) collected faecal samples from 767 chickens selected randomly in May or October 2011. In addition, 110 chickens were sampled in both May and October. Out of 767 faecal samples tested *Eimeria* oocysts were detected microscopically in 427 (56%, 95% confidence interval (95% CI) 52-59%). In a subset of samples further analyzed, seven species of *Eimeria* were detected by real-time PCR with the prevalence of some species varying by region.

The identification by morphometry and Polymerase chain reaction (PCR) of various *Eimeria* species were compared by Haug *et al.,* (2008). The samples were collected from feces and litter. After comparison between PCR results with the tentative identification based on morphometry showed complete agreement only in 49% (30 of 61) of the flocks.

To detect, identify and differentiate various pathogenic species of *Eimeria* in poultry, Schnitzler *et al.* (1998) described a polymerase chain reaction (PCR)-based assay. Sequencing the internal transcribed spacer 1 (ITS1) regions of ribosomal DNA (rDNA) from *Eimeria acervulina, E. brunetti, E. necatrix*, and *E. tenella* was performed and regions of unique sequences were identified.

A PCR -based study was done for the detection of *Eimeria acervulina* by Molloy *et al.* (1998). Primers were designed to amplify a fragment of the EASZ240/160 sporozoites antigen gene. The PCR assay detected a minimum of 10 *E. acervulina* oocysts in a mixed population containing a total of 106 oocysts and from their studies they concluded that traditional methods for diagnosis of coccidial infections do not reliably differentiate between species. When there is a mixed infection, only the major species present in the sample are likely to be identified. The PCR assay detects *E. acervulina* oocysts present as 0.001% of the total population.

Seventeen fecal samples were collected by Jordan *et al*. (2018) from 6 broiler farms and two pluck shops, showing clinical signs of coccidiosis in Trinidad. His study by using qPCR revealed the presence of five *Eimeria* species (*E. acervulina, E. maxima, E. mitis, E. necatrix, and E. tenella*).On all broiler farms, mixed infections were detected and DNA of two highly pathogenic *Eimeria* species (*E. tenella and E. necatrix*) was detected in feces collected from clinically ill birds, sampled from the two pluck shops.

Carvalho *et al*. (2011) identified the seven species of the genus *Eimeria* in all broiler farms in the micro-region of Feira de Santana with help of the PCR technique. *Eimeria brunetti* with less frequency (16.7%) was recorded by him. While all farms (100%) were positive for both *Eimeria maxima* and *Eimeria praecox*.

Chere *et al.* (2022) collected and examined fresh faecal samples from 50 small and large-scale farms in different regions of Ethiopia. He used a PCR-based assay for the differentiation of *Eimeria* oocysts. The extracted DNA templates & genus-specific primers were used for amplification of ITS-1 region from 7 *Eimeria spp*. of chicken and he recorded that all 7 species were present in both large and small-scale farms. He also revealed that mixed infections of *Eimeria spp*. per sample was common in most farms.

A total of 318 faecal samples from 137 broiler farms were examined by Geng *et al.* (2021) in Henan and Hubei parts of China. They used an internal transcribed spacer 1 (ITS1) sequence of ribosomal DNA to identify the species of *Eimeria*. Based on PCR results, they concluded that the positivity rate of *Eimeria* was 97.17% (309/318), and the most common species found are *Eimeriamitis* (66.67%), *E. tenella* (46.86%), and *E. necatrix* (41.51%).

2.8 Chemoprophylaxis:

The drugs which are used for the prevention and control of coccidiosis are known as anticoccidials. Coccidiocidal drugs destroy the *Eimeria spp*. while coccidiostatic drugs prevent the replication and growth of *Eimeria spp.* Preventive medication is always effective in the control of coccidiosis. In structured programs, these anticoccidials are very useful to obtain maximum production (Kant *et al.,* 2013).

Anticoccidials via feed also act as feed additives and have a great role in the poultry industry. It is easy and economically effective in commercial farms (Chapman 1997). They are categorized as polyether ionophoric compounds i.e. monensin, narasin, salinomycin, maduramycin, semduramycin, lasalocid, and

chemical or synthetic compounds i.e. sulphonamides, amprolium, diclazuril, nicarbazine, halofuginone, clopidol, dicoquinate, robenidine (Chapman, 1999; Allen and Fetterer, 2002).

Most of the agents exert their anticoccidial effect by acting on the schizonts or sometimes gamonts. Anticoccidial drugs are commonly used to control coccidiosis in poultry which led to the development of drug resistance. Resistance has been developed to all of the compounds that have been introduced and if chemotherapy is to remain the main method of control. When chemicals have been used extensively for the control of any disease drug resistance occurs and this is true in the case of coccidiosis (Chapman, 1997).

In recent years, due to the continuous development of drug resistance against anticoccidials concern is increased regarding the control of coccidiosis and alternative programs should be introduced without any effect on production (Giannenas *et al.,* 2003). Due to common use, the cell membrane permeability of *Eimeria* oocysts has been altered and resistance against principally used different anticoccidials like monensin, narasin, sulphonamides, amprolium, etc. has been developed (Abbas *et al.,* 2011).

Stephen *et al.* (1997), recorded multiple resistances by *Eimeria* isolates against monensin, nicarbazine, salinomycin, and maduramycin and also cross-resistance against monensin, maduramycin, and salinomycin. Partial resistance to salinomycin by *Eimeria* tenella was observed by Abbas *et al.,* (2008a) in Pakistan, similar findings were also noted by Yadav and Gupta, (2001) in North India.

Kawazoe and Fabio (1994) also recorded the resistance against diclazuril by isolates of *Eimeria acervulina* & *Eimeria maxima* in brazil. After the in-vivo & in-vitro studies, McManus *et al.* (1968) observed the resistance against quinolones by *Eimeria tenella, Eimeria brunetti, Eimeria maxima*, and *Eimeria acervulina*. Increased resistance against monensin to field isolates of coccidia was observed by Ruff *et al.* (1985).

Due to increasing resistance to existing anticoccidials, several natural products and plant derivatives can be used to control coccidiosis in poultry (Allen and Fetterer, 2002; Abbas *et al.,* 2011). To overcome the resistance against chemical and synthetic agents, the use of plant extracts like essential oils is the best alternative to control coccidiosis (Chapman, 1997). Plant derivatives and essential oils can be used as alternatives to antiparasitic drugs (Anthony *et al.,* 2005). Natural substances can be used to destroy *Eimeria* oocysts (Quiroz and Gonzalez, 2015).

2.9 To study the efficacy of the essential oil of Thymol as an anticoccidial agent in broilers:

Control of coccidiosis is achieved by good management, vaccines, using natural feed additives, and anticoccidial drugs. Prevention of disease by using essential oils instead of chemical or synthetic agents is called aromatherapy, which is closely related to phytotherapy. It includes the use of distilled plant volatiles. Essential oils differ in chemical composition from other herbal products as the distillation process only recovers lighter phytomolecules, (Peek and Landman, 2011).

Essential oils supplementation shows a precise reduction in *Eimeria* oocyst excretion and positive effects on intestinal lesion scores in chicks (Zhai *et al.,* 2018). *Rosmarinus officinalis* & *Thymus vulgaricus* are rich in phytochemicals like thymol, and carvacrol and they can be used to prevent & treat coccidiosis in birds (Lahlou *et al.,* 2021). Significant anticoccidial effects have been exerted by, thyme and their phenols, such as thymol and carvacrol (Ibrir *et al.,* 2001).

Remmal *et al.* (2011) conducted an in vitro study to check the ability of essential oils to destroy *Eimeria* oocysts. They used microscopic counting of oocysts and 273 nm absorbing material release. The study was carried out on ten different essential oils in a liquid medium and they recorded that Artemisia, tea tree, thyme, and clove EOs were the most effective. The treatment

of *Eimeria* oocyst with these EOs leads to their lysis. After approximately three hours of contact, these results were obtained.

To check in vitro anticoccidial effects of eight different essential oils, Remmal *et al.* (2013) experimented with oocysticidal activity in a liquid medium. After the screening of the eight EO components, it was observed that the number of oocysts decreases after the treatment at a concentration ranging between 0.3 and 20 mg/ml. They recorded that the most effective components are carvacrol followed by carvone, isopulegol, thymol, and eugenol at a concentration of less than 2 mg /ml.

A study was carried out by Giannenas *et al.* (2003) to check the effect of dietary supplementation of oregano essential oil on the performance of 120 broilers challenged with *Eimeria* tenella at 14 days of age. After 42 days of study, he observed the survival rate, lesion score, and OPG count and recorded that oregano essential oil exerted an anticoccidial effect against *E. tenella,* which was, slightly lower than that of lasalocid.

A trial was conducted by Rondon *et al.* (2006) to evaluate the effect of two specific EOs in broilers challenged with *Eimeria* infection. They carried out this trial in both vaccinated and unvaccinated groups of broilers. A powder form of the EO blend was added at 100 ppm to the basal diet. The main compounds of the EO blend are Thymol, Eugenol, Curcumin, & Piperin. After a comparison of results, they concluded that EO blends show effectivity against mixed infection of *Eimeria* and it could be used as an alternative to ionophores in case of drug resistance.

Asli and Rashti (2015) experimented to check the effects of oregano essential oil on coccidiosis prevention along with growth performance in mildly challenged broilers. They fed oregano essential oil at the rate of 300 ppm & 500 ppm in feed. On the 22nd day, birds were challenged with the LIVACOX-T vaccine. After slaughtering birds' OPG count, intestinal lesion scores were recorded, and based on their recordings concluded that oregano oil

supplementation at the dose of 500 ppm in the diet has a beneficial effect on the prevention of coccidiosis.

A study was carried out by Tsinas *et al.* (2011) to examine the efficacy of oregano essential oil at the rate of 300-600mg/kg supplementation in diet on the performance of coccidiosis-challenged broiler chickens at 14 days of age. The results were compared with the control group treated with salinomycin @60mg/kg. The results indicated that oregano essential oil supplementation in diet exerted an anticoccidial effect.

Scheurer *et al.* (2013) carried out a study on 1080 broiler chicks to examine anticoccidial effects of 3 phytochemicals viz. Oregano@2 kg/t, combination of saponin, curcuma & inulin @1kg/t, Quilaja @1kg/t. Chicks were challenged with *E. acervulina, E. maxima* & *E. tenella* on the 15th day via feed & results were compared with Narasin @0.7kg/t. After recording the results they concluded that none of the products was effective at using dosage.

The in-vitro activity of thymol, carvacrol, and saponins as anticoccidials was checked in vitro model of coccidiosis by Felici *et al.* (2020). Sporozoites of *Eimeria spp.* were collected from field samples and used for 2 different invasion assays on Madin-Darby Bovine Kidney cells (MDBK). They challenged cells with 5 x 104 sporozoites and treated them with saponin (10 ppm), thymol, and carvacrol (7 ppm each). A combination of thymol, carvacrol, and saponin was also used for some groups at two different groups. After recording overall observations, the study concluded that invasion of *Eimeria* sporozoites in MDBK cells is inhibited in presence of thymol, carvacrol, and saponins, which indicated the anticoccidial effect of these compounds.

Bozkurt *et al.* (2016) checked the effect of oregano essential oil as anticoccidial in comparison with Monensin sodium, in feed supplements. All the birds were challenged with *Eimeria spp*. at the 12th day of age. After the 42nd day, the observations were recorded & they concluded that oregano essential oil supported the intestinal absorptive capacity of birds. They also observed that oregano showed slight activity against the reproductive potential of *Eimeria.*

The efficacy of essential oil of thymol& eucalyptus on pigeon coccidiosis was investigated by Arafa *et al.* (2020). They conducted both in-vivo & in-vitro studies. After 72hrs incubation in the in-vitro trial, they observed that thymol at concentrations ≥1.25 % caused significant changes in sporulated and unsporulated oocysts. Eucalyptus showed activity at both 5 and 10 % concentrations only on unsporulated oocysts. Birds were orally infected by 25 x 103 sporulated oocysts of *Eimeria labbeana* during in-vivo trials & treated with Thymol @40mg/kg BWT via feed for 15 days. He observed that thymol minimized the adverse effect of coccidiosis with less severity of clinical signs, less OPG count, and improved body weight as compared to untreated infected birds. After all findings, it was concluded that thymol can be used to control pigeon coccidiosis as an alternative natural compound.

To evaluate the anticoccidial effect of herbal extract blend through feed containing garlic (*Allium sativum*), sage (*Salvia officinalis*), echinacea (*Echinacea purpurea*), thyme (*Thymus vulgaris*), and oregano (*Origanum vulgare*) extracts in broilers challenged with 170.000 sporulated oocysts of *Eimeria acervulina*, *E. tenella, E. maxima,* and *E. necatrix* at 12 days of age, Włosek and Swiatkiewicz (2012) experimented. Diet supplemented with 1gm/kg of herbal extract with equal contribution of each product. After recording the OPG count, lesion scoring, and other observations on the 42nd day they finally concluded that herbal extract was not significant at the given dose against coccidiosis.

Alp *et al.* (2012) studied 1200 broiler birds to evaluate the growth performance & anticoccidial effects of dietary oregano essential oil. The experiment was conducted in three groups with 5 replicates each. For treatment group's diet was supplemented with dietary oregano essential oil at a dose of 300mg/kg of feed. OPG was conducted after every 10 days. Results were recorded on the 42nd day which indicated that dietary oregano essential oil effectively lowered OPG count & also improved growth performance.

As per study and observations on coccidiosis-challenged broilers treated with essential oils of eucalyptus peppermint via drinking water at 0.69 ml/Kg

body weight, Barbour *et al.* (2015) concluded that both essential oil helps to control coccidiosis with reduced OPG count and intestinal lesion scores.

MATERIALS AND METHODS

During the study period, faecal samples were collected in a plastic container from broiler farms in and around the Nagpur region and brought to the laboratory. Samples were processed by sedimentation technique, and the last drop of sediment was checked under the low power of a compound microscope (10x) for the presence of *Eimeria* oocysts. Sediments from positive samples were sporulated by using 2.5% potassium dichromate.

Using these sporulated oocysts group of broiler birds was challenged for *Eimeria* infection, and in-vivo anticoccidial activity of essential thymol oil was checked. Some of the sporulated oocysts were separated by flotation technique for morphometric observations. A polymerase chain reaction was performed to confirm *Eimeria* species, as PCR gives the most sensitive and accurate results.

3.1 Materials

3.1.1 The following chemicals, reagents, and equipment were used

A. Laboratory chemicals and reagents

All the laboratory chemicals and reagents were of pure-molecular grade from Sigma, Hi-Media, and other national and international firms during the study period. The reagents for the research work were prepared in distilled water using molecular-grade water. The chemicals and reagent includes-

- Agarose (Invitrogen Bioservices India Pvt. Ltd Bangaluru)
- DNA extraction kit for stool (HIMEDIA HiPurAR Blood Genomic DNA Miniprep Purification Kit)
- DNA ladder (Promega)
- Ethanol(Hi-Media Laboratories India Pvt. Ltd. Nashik)
- Ethidium bromide (Sigma)
- Master Mix (Go Taq Green MM Promega)
- Oligonucleotide primers (Eurofins Genomics India Pvt., Ltd Bangaluru)
- Sodium chloride 99% (KR chemicals, India)

- Potassium Dichromate (Hi-Media Laboratories India Pvt. Ltd Nashik)

B. Sterilization of Glassware

The glassware (Borosil) was used and sterilized in a hot air oven to prevent contamination, as per the standard procedures.

C. Sterilization of Plasticware

Plastic ware used, including Eppendorf tubes, centrifuge tubes, microcentrifuge tubes, micro tips, and PCR tubes, were autoclaved to prevent contamination. For DNA isolation, collection tubes, Mini columns, Elution tubes, and DNA extraction kits were provided by QIAGEN (QIAamp Fast DNA Stool Mini Kit).

D. Equipment

In the present study, the commonly used equipments were –

- Autoclave pressure steam sterilizer.
- Compound light microscope (Lawrence and Mayo Pvt. Ltd Mumbai)
- Cooling microcentrifuge (Remi C-24 Plus)
- Electrophoresis unit (GENEI Pvt. Ltd Bangaluru)
- Gel documentation system (Gel Pro analyzer, SYNGENE International Limited).
- Water bath (Tempo Pvt. Ltd. Bombay volts)
- Thermal cycler (Applied biosystem, Thermo Fisher Scientific India Pvt. Ltd Mumbai)
- Spectrophotometer (Eppendorf)
- Vortex shaker (Macro scientific work, Delhi)
- Weighing balance (KERN, Germany)
- Spinwin (Tarsons) 32
- Deep Freezer (Qingdao Haier Biomedical)
- Microwave oven (LG)
- Sedimentation Flask (Borosil)

E. Materials/chemicals for field trial

- The powdered form of Thymol
- Maduramycin
- Sample collection bottles (plastic)

3.2 Methods:

3.2.1 Collection of Litter/Faecal Samples

Litter/faecal materials were collected from each poultry house at different spots following a roughly W-shaped path starting and finishing in the corners of one of the long sides of the house. Along this path, portions of the litter/faecal materials were manually collected, placed in sterile containers, and transported on an ice pack to the department of Veterinary Parasitology, NVC, Nagpur.

3.2.2 Laboratory Processing of the Samples.

In the laboratory, 200g of each sample was weighed, transferred into a plastic beaker, homogenized using a glass rod, and soaked in about 500 ml of distilled water overnight. The soaked samples were homogenized by thoroughly stirring using a glass rod and filtered through a metal sieve. The filtrate from each sample was allowed to sediment for about an hour on the laboratory bench, after which the supernatant fluid was discarded into a clean beaker. About 10 ml of the sediment was then transferred into a centrifuge tube and tested for the presence of *Eimeria* oocysts using the simple floatation technique described by Soulsby (1982).

3.2.3 Separation of oocysts:

Oocysts were separated from faecal material by flotation technique using saturated sodium chloride solution as described by Kumar *et al.* (2014) and Qi *et al.* (2020) with some modifications. Oocyst pellets were washed with tap water 6-7 times by centrifugation to remove salt material.

3.2.4 Morphometric identification:

Separated oocysts were observed under a microscope for further morphometric studies, including size, shape, measurements, etc., as per suggestions by Duszynski and Wilber (1997) and McAllister and Upton (1989).

3.2.5 Molecular detection and species identification:

Extraction of genomic DNA from separated oocysts:

For genomic DNA isolation, oocysts were ruptured with the help of 3mm glass beads, as the process suggested by Cha *et al.* (2014) and Bawer *et al*. (2021), with certain modifications. Distilled water (0.25ml) was added to the washed oocyst pellet sample with an equal volume of glass beads in 2 ml Eppendorf tubes. After dilution with 0.25ml distilled water concentration of oocysts was checked under a microscope, and samples with more than 100 oocysts per 10µl were preferred for DNA extraction for better concentration of DNA. Eppendorf tubes, along with oocyst pellet, distilled water, and glass beads, were vortexed at high speed for 20-30 minutes, and ruptured oocysts were observed under a microscope for confirmation. Glass beads were separated, and ruptured oocysts were used for isolation of DNA. DNA was isolated by using QIAamp Fast DNA Stool Mini Kit.

1) Procedure for DNA extraction:

1. 0.25 g of feces or 0.5 g of the centrifuged sewage pellet into a bead-beating tube was weighted. Samples were kept on ice until InhibitEX Buffer was added to reduce DNA degradation risk.
2. InhibitEX (1 ml) Buffer was added to the tube containing the sample aliquot.
3. Vortexed continuously until the sample is thoroughly homogenized
4. Sample was treated in a Tissue Lyser at 30 Hz for 3 x 30 s, with cooling on ice between each treatment.
5. Heated the sample at 95℃ for 7 min.
6. Vortexed the sample for 15 seconds.

7. The sample was centrifuged at full speed for 1 min to pellet sample particles.
8. Proteinase K (30 µl) pipetted into a new 1.5 ml microcentrifuge tube (not provided).
9. Supernatant from step 7 was pipetted (400µl) into the 1.5 ml microcentrifuge tube containing proteinase K.
10. Buffer AL (400µl) was added and vortexed for 15 seconds.
11. Incubated at 70°C for 10 min. Centrifuged briefly to remove drops from the inside of the tube lid.
12. Ethanol (96–100%) was added (400µl) to the lysate and mixed by vortexing. Centrifuged briefly to remove drops from the inside of the tube lid.
13. Lysate from step 12 was carefully applied (600µl) to the QIAamp spin column. Closed the cap and centrifuged at full speed for 1 min, closed each spin column to avoid aerosol formation during centrifugation. If the lysate was not completely passed through the column after centrifugation, samples were centrifuged again until the QIAamp spin column was empty.
14. Remaining lysate from step 12 to the spin column was applied, closed the cap, and centrifuged at full speed for 1 min. Placed the spin column was in a new 2 ml collection tube, and the tube containing the filtrate was discarded. Each spin column was closed in order to avoid aerosol formation during centrifugation; if the lysate had not completely passed through the column after centrifugation, centrifuged again until the QIAamp spin column was empty. •
15. Carefully opened the QIAamp spin column, and 500 µl Buffer AW1 was added and centrifuged at full speed for 1 min. Placed the QIAamp spin column in a new 2 ml collection tube and discarded the collection tube containing the filtrate.

16. Carefully open the QIAamp spin column and add 500 µl Buffer AW2. Centrifuge at full speed for 3 min. Discard the collection tube containing the filtrate.
17. Place the QIAamp spin column in a new 2 ml collection tube (not provided) and discard the old collection tube with the filtrate. Centrifuge at full speed for 3 min. This step helps to eliminate the chance of possible Buffer AW2 carryover.
18. Transferred the QIAamp spin column into a new, labeled 1.5 ml low-DNA bind microcentrifuge tube and pippeted 100 µl or 50 µl Buffer ATE directly onto the QIAamp membrane. Incubated for 3 minutes at room temperature, then centrifuged at full speed for 1 min to elute DNA. Pippeted another 50 µl Buffer ATE directly onto the same QIAamp membrane. Incubated for 2 min at room temperature, then centrifuged at full speed for 1 min. To elute DNA. This step combines the DNA-containing eluate from 2x50 µl Buffer ATE.
19. QC the sample and isolated DNA amount has been quantified.
20. The extracted DNA was stored at -20°C or -80°C until further use.

ii. DNA amplification of *Eimeria spp*. by Polymerase Chain Reaction

Genomic DNA extracted from *Eimeria* oocysts was amplified. Primers used to amplify the ITS-1 region of specific *Eimeria spp*. and universal genus-specific DNA primers targeting COI genomic region are described in table 3.1.

The universal primer used was designed by Schwarz *et al.* (2009), and the species-specific primers of *Eimeria tenella, Eimeria necartix, Eimeria acervulina*, and *Eimeria brunetti* were designed by Schitzler *et al.* (1998), *Eimeria maxima* by Lew *et al.,* (2003), *Eimeria praecox* and *Eimeria mitis* by Schitzler *et al.,(*1999).

Table 3.1: List of primers used, their sequences, and amplicon size.

Sr. no.	Gene	Species	Primer seq (5' to 3')	Amplicon size (bp)
1	COI	Universal primer for *Eimeria spp.*	F- GTTTGGTTCAGGTGTTGGTTG R- ATCCAATAACCGCACCAAGAG	810
2	ITS-1	*Eimeria tenella*	F- AATTTAGTCCATCGCAACCCT R- CGAGCGCTCTGCATACGACA	278
3	ITS-1	*Eimeria necatrix*	F- TACATCCCAATCTTTGAATCG R- GGCATACTAGCTTCGAGCAAC	285
4	ITS-1	*Eimeria acervulina*	F- GGCTTGGATGATGTTTGCTG R- CGAACGCAATAACACACGCT	321
5	ITS-1	*Eimeria maxima*	F- CWCACCACTCACAATGAGGCAC R- GTGAWTCGTTYGRRAGTTTGC	145
6	ITS-1	*Eimeria praecox*	F- CATCATCGGAATGGCTTTTTGA R- AATAAATAGCGCAAAATTAAGCA	368
7	ITS-1	*Eimeria mitis*	F- TATTTCCTGTCGTCGTCTCGC R- GTATGCAAGAGAGAATCGGGA	306
8	ITS-1	*Eimeria brunetti*	F- GATCAGTTTGAGCAAACCTTCG R- TGGTCTTCCGTACGTCGGAT	311

Table 3.2: The cyclic conditions for a PCR reaction with Universal primer, *Eimeria brunetti*, and *Eimeria tenella* species-specific primers were as follows:

Steps	Temperature and time	No. of cycles
Steps 1 - Initial denaturation	94˚C for 5 min	1
Step 2- Denaturation	94˚C for 30 seconds	30
Steps 3- Annealing	55˚C for 30 seconds	
Steps 4- Extension	72˚C for 2 min	
Steps 5 - Final Extension	72˚C for 10 min	1
Hold	4˚C for ∞	

Table 3.3: The cyclic conditions for a PCR reaction with *Eimeria acervulina* and *Eimeria mitis* species-specific primers were as follows

Steps	Temperature and time	No. of cycles
Steps 1 - Initial denaturation	94˚C for 5 min	1
Step 2- Denaturation	94˚C for 30 seconds	30
Steps 3- Annealing	54˚C for 30 seconds	
Steps 4- Extension	72˚C for 2 min	
Steps 5 - Final Extension	72˚C for 10 min	1
Hold	4˚C for ∞	

Table 3.4: The cyclic conditions for a PCR reaction with *Eimeria necatrix* and *Eimeria praecox* species-specific primers were as follows

Steps	Temperature and time	No. of cycles
Steps 1 - Initial denaturation	94˚C for 5 min	1
Step 2- Denaturation	94˚C for 30 seconds	30
Steps 3- Annealing	50˚C for 30 seconds	
Steps 4- Extension	72˚C for 2 min	
Steps 5 - Final Extension	72˚C for 10 min	1
Hold	4˚C for ∞	

Table 3.5: The cyclic conditions for a PCR reaction with *Eimeria maxima* species-specific primers were as follows:

Steps	Temperature and time	No. of cycles
Steps 1 - Initial denaturation	94˚C for 5 min	1
Step 2- Denaturation	94˚C for 30 seconds	30
Steps 3- Annealing	53˚C for 30 seconds	
Steps 4- Extension	72˚C for 2 min	
Steps 5 - Final Extension	72˚C for 10 min	1
Hold	4˚C for ∞	

The PCR reaction mixture was carried out in a final reaction volume of 25µl containing 12.5µl Master mix, 1µl Forward and Reverse primers, 5µl DNA template, and 5.5µl Nuclease free water. At every PCR setup, a negative control was run along with the samples.

Table 3.6: The PCR reaction was as follows

Components	Quantity(µl)
Master mix	12.5
Reverse primer	1
Forward primer	1
Nuclease free water	5.5
DNA template	5
Total	25 µl

iii. Agarose gel electrophoresis:

For the confirmation of the quality of DNA and PCR, amplified products were subjected to agarose gel electrophoresis submarine horizontal gel electrophoresis apparatus.

iv. Preparation of agarose gel:

Agarose powder 0.40 gm was added to 40 ml of 0.5X TAE (Tris-acetate EDTA) buffer to prepare 1% of agarose gel. This mixture was kept in a microwave oven till it became crystal clear, and ethidium bromide was added 4µl.

v. Agarose gel casting:

The agarose gel was poured into the casting tray with a comb fixed in it. The gel was kept at room temperature for at least 40-45 minutes. After the polymerization of the gel, the comb was carefully removed without disturbing the wells. The tray with the gel was transferred to an electrophoresis tank containing 0.5XTAE buffer. A sufficient buffer was added to cover the gel to a depth of at least 1mm.

vi. PCR product loading:

The PCR product of 7 µl was loaded in each of the wells along with the DNA ladder (100 bp) was loaded in the initial well. Nontemplate control using nuclease-free water was loaded in the last well. The samples were run at 90 volts for 45 min till the dye reached two-thirds of the gel.

vii. Visualization of gel:

The gel was observed under the UV transilluminator Gel Documentation System (Gel Pro analyzer, SYNGENE International Limited).

viii. Purification of PCR products:

The PCR products were purified using QIAqucik PCR Purification Kit (Germany) following the manufacturer's protocol. The stored PCR products were thawed and mixed at 25˚C, and 46 µl of binding buffer was added to 46 µl of the PCR product in the tube and mixed until the mixtures turned yellow. The solutions were then placed in QIAquick purification columns and centrifuged for 1 minute until the flow through the column and the flow-through was discarded. To the QIAquick column, 750 µl of wash buffer was added, centrifuged for 1 minute, and the flow-through was discarded. The empty QIAquick column was centrifuged again to remove residual wash buffer, then transferred to a clean 1.5 ml microcentrifuge tube. To elute the DNA, 50 µl of elution buffer was added to the columns and centrifuged for 1 minute, the flow-through was discarded, and the eluted purified DNA was then stored at -20°C.

ix. Sequencing of PCR product & analysis of Nucleotide sequence by Basic Local Alignment Search Tool (BLAST).

Purified DNA products positive for *Eimeria acervulina, Eimeria tenella,* and *Eimeria mitis*, along with 10µl of each primer targeting the amplicon size 321 bp, 278 bp, and 306 bp, respectively, were sent to Eurofins Genomics India Pvt. Ltd. Bangalore, Karnataka, India for sequencing. Sequencing was carried out through a 3730XL DNA Analyzer and 96 capillaries from Life

technologies. Sequences were taken through Codon Code Aligner. The ab1 file of forward and reverse sequences were opened in Codon Code Aligner ends were clipped and assembled with processing, then trimmed all bases to the left and right, and discrepancies were auto-edited bases to match consensus and aligned using multiple alignment program ClustalW. MegaBLAST searches were done in GenBank to identify matches to the sample sequences. ITS-1 sequences from the GenBank with 98-100 percent or closest similarity to the sample sequence were considered. The sequences were submitted to the NCBI gene bank.

x. Phylogenetic analysis:

The sequences were analysed phylogenetically. The phylogenetic analysis was performed using the ITS1 region sequence of studied and reference isolates. The sequences were aligned using ClustalW in MEGA7, Kumar *et al*. (2016). The sequences were trimmed for unaligned ends, and a maximum likelihood phylogenetic tree was constructed in RAxML version-8 with 100 bootstraps, Stamatakis (2014). The tree was visualized and annotated in MEGA7.

3.2.6 Anticoccidial effect of essential oil of Thymol

A total of 90-day-old broiler chicks (VENCOBB430) were obtained from a commercial hatchery and were randomly divided into three groups consisting of 30 birds each. Every group was further divided into two replicates of 15 birds each. They were then housed in floor pens (5 m^2) equipped with litter material.

Infection of coccidiosis was sprayed on the floor, and then litter material was bedded. The experiment was conducted in July and August. Diets were formulated as per standards excluding coccidiostat, antibiotics, and probiotics to check the effect of Thymol as anticoccidial. The negative control diet contained no coccidiostat or Thymol. In contrast, the other two diets were supplemented with the powdered form of either thymol essential oil at a level of 20gms/100kg or Maduramycin at the level of 50gms / 100kg feed.

The commercial thymol oil was in powder form. The pre-starter, starter, and finisher diets were provided from 1 to 10 days, 11 to 22 days, and 23 to 42

days of age, respectively. Feed and water were provided at all times. The body weights of the birds were measured individually, and feed intake per pen was recorded weekly. Mortality was recorded as it occurred. Total feed consumption per bird was adjusted according to daily mortalities.

Fresh droppings from each group were collected at an interval of 7 days till the end of the trial to carry out the OPG count. The OPG count was carried out by using McMasters chamber as per suggested by Hodgson (1970) with slight modifications.

On the 28th and 42nd days, three birds from each group were sacrificed to check the intestinal lesions, and the severity of coccidial lesions was scored from 0 to 4, as described by Johnson and Reid (1970). Tissue samples from the intestine showing gross lesions were collected for histopathology in 10% formalin. After fixation, these tissues were processed using xylene and alcohol followed by impregnation in paraffin wax as per routine method, and 5μ sections were cut and stained with H&E as per described by Luna(1968) for recording histopathologic observations under light microscopy.

RESULTS & DISCUSSION

The present study was carried out at PRTC, Nagpur, and the Department of Veterinary Parasitology, NVC, Nagpur. Experimental infection was done in broilers reared at the PRTC, Nagpur. To know the species of coccidia infecting the broilers in and around Nagpur, 30 commercial broiler farms were screened during the study period.

Droppings and litter material from these farms were collected and brought to the Department of Veterinary Parasitology laboratory. The samples were subjected to sedimentation, and oocysts were identified. The oocysts were separated as per the method given by Kumar *et al.* (2014) and Qi *et al.* (2020) and subjected to morphometry and molecular analysis. The results of the present study are summarized and discussed as given below:

4.1 Prevalence of Coccidiosis:

Out of 30 farms screened, 21 (70%) were found positive for coccidiosis infection, out of which 3(14.28%) were positive only for *Eimeria tenella*, 11 (52.28%) showed mixed infection with *Eimeria acervulina*, *Eimeria mitis*, and *Eimeria tenella* and 7(33%) showed mixed infection with *Eimeria acervulina* & *Eimeria mitis.* A graphical presentation of prevalence is shown in (Fig. 4.1 & 4.2).

The results are in agreement with Rao *et al*. (2012) that in cases of mixed infections most common species observed are *Eimeria acervulina*, *Eimeria maxima, Eimeria mitis*, and *Eimeria tenella*. Kalita *et al.* (2021) also recorded these four species as prevalent, but *Eimeria acervulina* & *Eimeria tenella* are commonly found in mixed infections.

Overall prevalence recorded by Pant *et al.* (2018); Kumar *et al.* (2015); Rao *et al.*(2012); Oljira *et al.* (2012), Mohammad *et al.* (2002); Shirzad *et al.* (2011) is nearly similar to prevalence observed in this study. Nikam *et al.* (2012);

Sharma *et al.* (2013); Kala *et al.* (2013); Jadhav and Nikam (2014); Gharekhani *et al.* (2014) recorded lesser incidence of coccidiosis in their respective study areas.

This variation in prevalence is might be due to different climatic conditions or the development of resistance, (Awais *et al.,* 2012). Ambient temperature (25^0C) and relative humidity greater than 60 % provide suitable environmental conditions for the sporulation of oocysts and favour coccidiosis (Anderson *et al.,* 1976). The density of birds, type of litter material used, age, breed, season, management, and sanitation are responsible for the incidence of coccidiosis (Prakashbhau *et al.* 2017). The single or mixed infection of *Eimeria tenella* with other species is possible because of its predominant nature (Iqbal *et al.,* 2017).

4.2 Morphometric identification of *Eimeria* species:

The micrometry was carried out by using Zeiss Axio Lab A1 trinocular microscope and Zeiss Zen 3.2 (Blue edition) software.

Microscopic identification based on morphometric measurements and shape, as per Tyzzer (1929a), revealed the presence of *Eimeria mitis* (Plate 4.1)*, Eimeria acervulina* (Plate 4.2), and *Eimeria tenella* (Plate 4.3). The results of morphometry are summarized in table no. 4.1.

Table 4.1: Results of morphometry:

Species	Avg. dimensions (length x breadth)	Avg. Shape index (length/breadth)	Shape
Eimeria acervulina	19.70µm x 16.10µm	1.22	Ovoid
Eimeria tenella	22.92 µm x 19.78µm	1.15	Ovoid
Eimeria mitis	19.19 µm x 18.38µm	1.04	Spherical

In poultry, the overlapping of oocyst dimensions is commonly noticed. In chicken *Eimeria* species, there is the absence of a polar cap, indistinguishable micropyle, and oocyst wall structure (Joyner and Long, 1974). It is difficult to differentiate between *Eimeria acervulina* and *Eimeria mitis*, even though many characters have been developed to distinguish them (Long and Joyner, 1984).

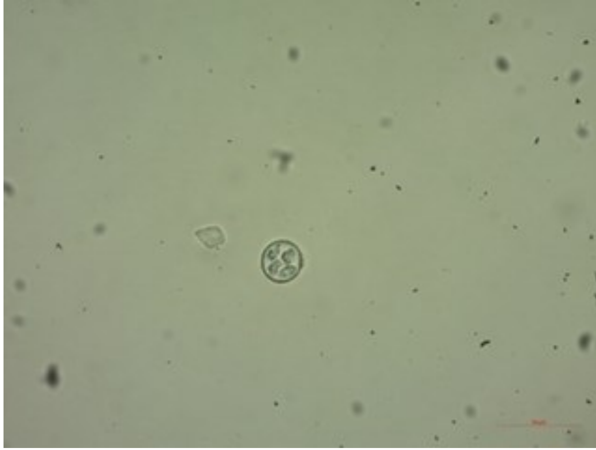
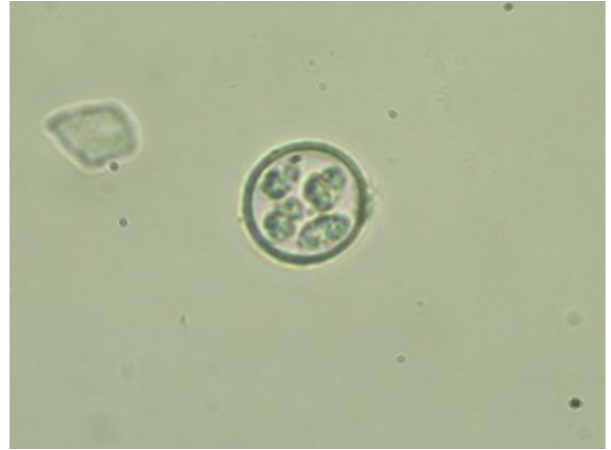

Plate 4.1: Sporulated oocyst of *Eimeria mitis* (400x) , (Zoom image on right side)

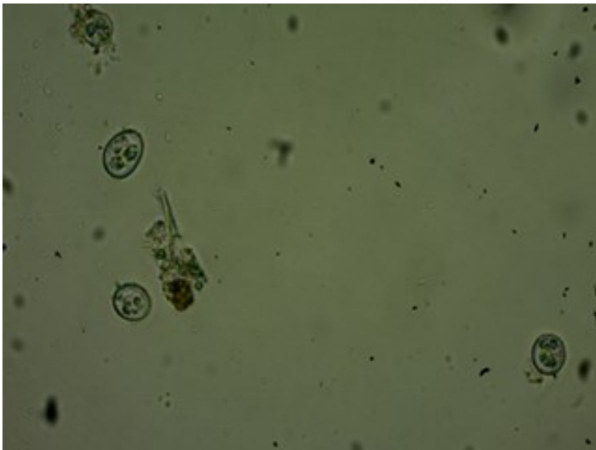
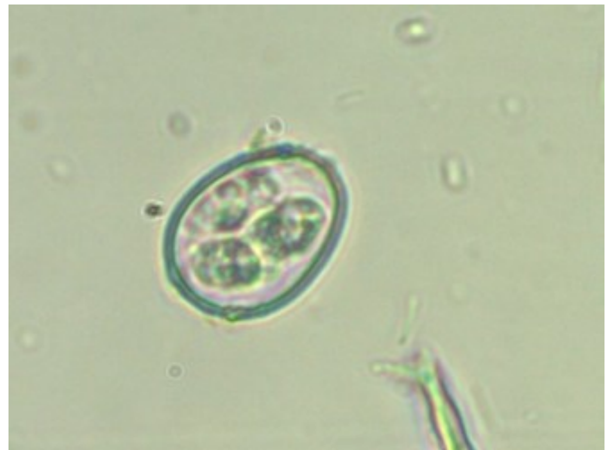

Plate 4.2: Sporulated oocyst of *Eimeria tenella* (400x) (Zoom image on right side)

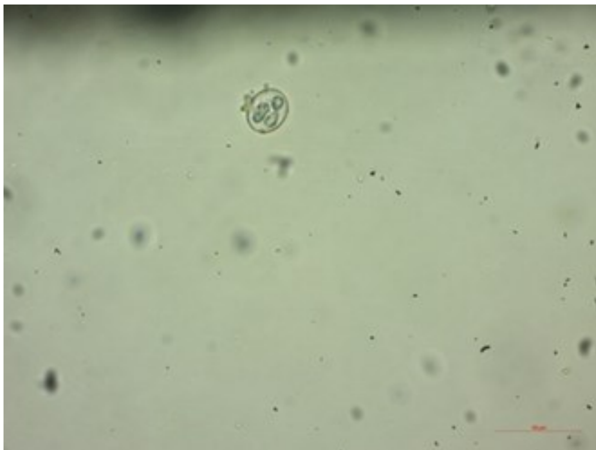
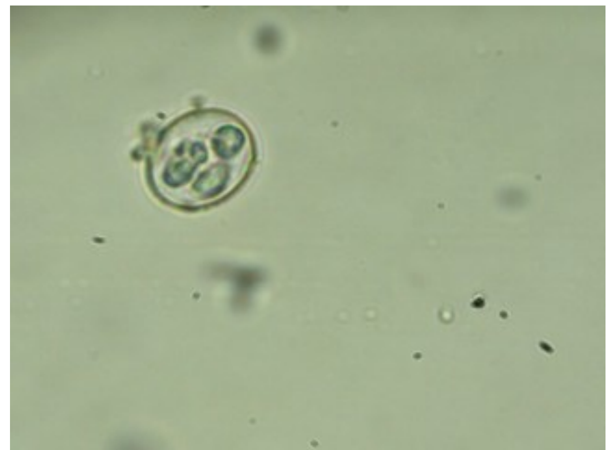

Plate 4.3: Sporulated oocyst of *Eimeria acervulina (400x)* (Zoom image on right side)

Fig: 4.1 Graph of prevalance of coccidiosis

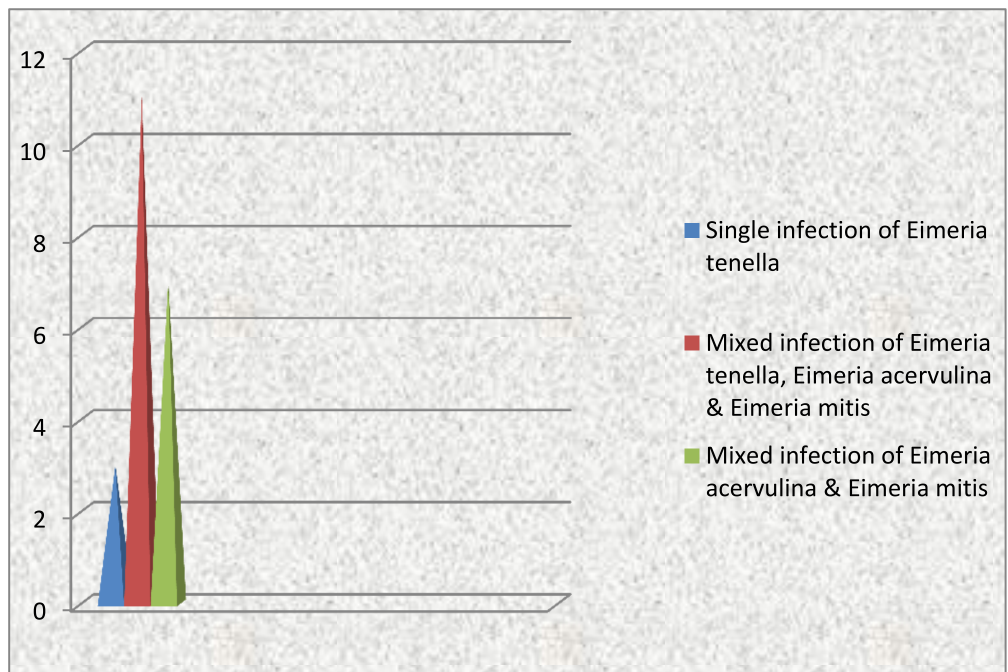

Fig: 4.2 Comparative species infection:

The shape of *Eimeria acervulina* and *Eimeria tenella* is ovoid egg-shaped, and *Eimeria mitis* is spherical (Tyzzer 1929b). Findings in this study ultimately agreed with this, but the size and dimensions for *Eimeria mitis* and *Eimeria tenella* observed are slightly greater. The shape index observed nearly matches the shape index recorded by Amer *et al.,* (2010) with a slight difference in the average dimensions of oocysts. Although *Eimeria mitis* is considered benign and does not kill the birds, it can cause economic losses commercially; hence its control is also important (Allen and Fetterer, 2002).

Thebo *et al*. (1998) recorded the spherical shape of *Eimeria mitis* with 17 µm average dimensions, while Nikam *et al.* (2012) recorded 14.8 µm x 15 µm, which are smaller than the observations recorded in the present study. Kucera (1990) categorized *Eimeria acervulina* and *Eimeria mitis* within the same group with 18µm average dimensions, which was nearly similar to this study. Morphometric dimensions of *Eimeria acervulina* and *Eimeria tenella* are moderately greater than recordings by Iqbal *et al.* (2017), but the shape index was similar.

4.3 Molecular identification of *Eimeria* species:

4.3.1 Identification of Eimeria spp. by using PCR and gel electrophoresis

As the morphometric identification of species is less sensitive and requires expertise, the confirmatory species diagnosis of *Eimeria*, the most sensitive and effective technique PCR, was used. The separated oocysts were used for the isolation of DNA by using QIAamp Fast DNA Stool Mini Kit as per the manufacturer's instruction.

The primers used for PCR amplification were designed by Schwarz *et al.* (2009), Schitzler *et al.* (1998), Lew *et al.* (2003), and Schitzler *et al.* (1999). Species-specific primers target the ITS-1 gene, and universal primers target the COI gene. 1% agarose gel was used to run the amplified PCR product. PCR amplification of the COI gene of universal primer showed a predominant band of *Eimeria* genus according to the marker size at 810bp, and amplification of ITS-1

region of genomic oocyst DNA by using species-specific primers confirmed the presence of three species i.e. *Eimeria acervulina* (321bp), *Eimeria mitis* (306bp) & *Eimeria tenella* (278bp) (Plate 4.6).

The use of saturated salt in the flotation technique for separation of oocysts and glass beads for mechanical rupture of oocysts (Plate 4.5).Before isolation of DNA resulted in a very good concentration of DNA and increased PCR sensitivity, which entirely agrees with the methods suggested by Kumar *et al.* (2014) & Bawer *et al*. (2021).

4.3.2 Sequence analysis:

The purified PCR products were sequenced using commercially available services. The edited sequences were blasted in GenBank and identified by comparing them with the related sequences of the ITS 1 & COI gene. Sequences that showed the highest similarity were considered the closest matches to the sequences of this study and were downloaded from the database for phylogenetic analysis.

The ITS 1 and COI gene sequences obtained in this study were aligned with the published sequence in the database and showed 99%-100% homology with reference sequences, except *Eimeria mitis* showed 94% homology. Table 4.2 shows the sequences submitted to the NCBI database with GenBank accession numbers.

Alignment of query *Eimeria tenella* (Universal primer COI gene) and reference sequence from GenBank; the query fully aligned (100%) with the reference sequence of *Eimeria tenella* strain Ingten mitochondrion (Ref. Seq. ID KX094951.1) Shown in (Fig. 4.3).

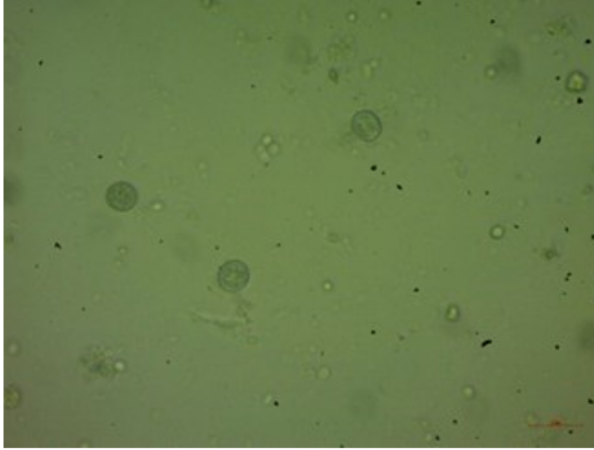
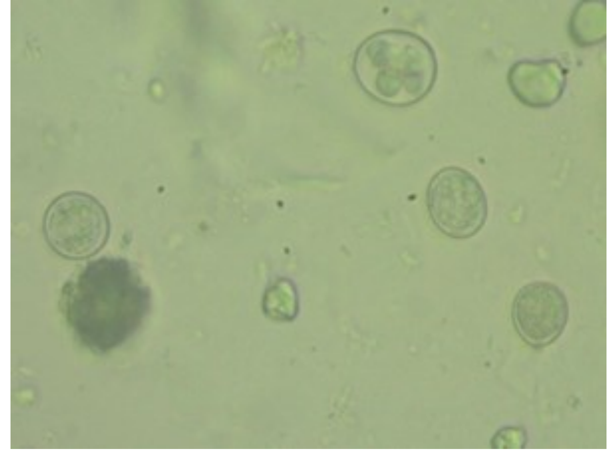

Plate 4.4: Unsporulated oocysts of *Eimeria spp.* (400x)

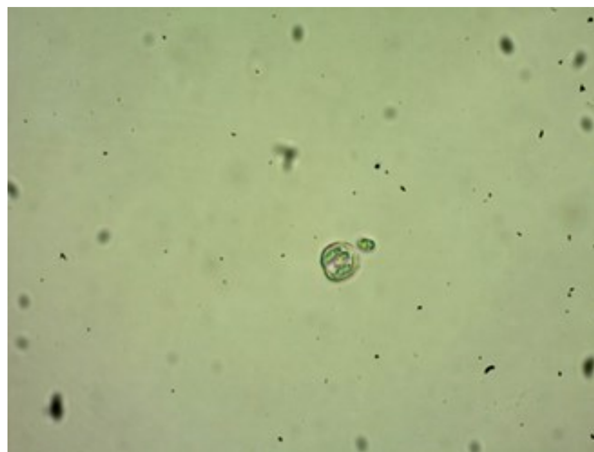

Plate 4.5: Ruptured oocyst by using glass beads *(400x)*

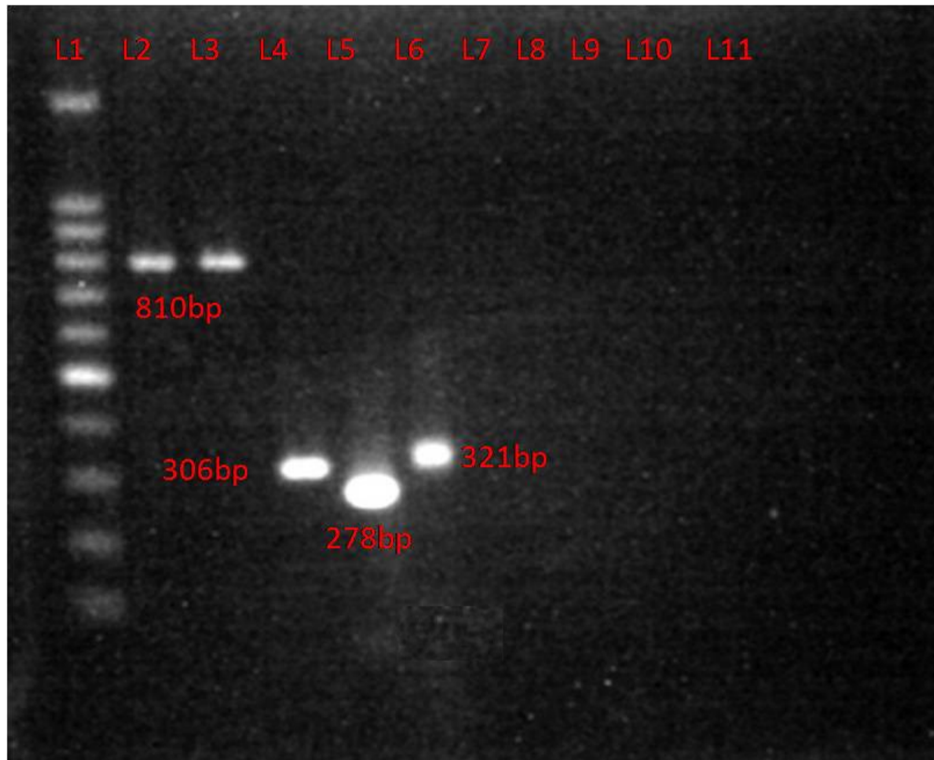

Plate 4.6: Sample agarose gel electrophoresis of PCR products

Lane 1: 100 bp DNA ladder
Lane 2: Positive control
Lane 3: *Eimeria* genus confirmation with universal primer.
Lane 4: Positive sample for *Eimeria mitis*
Lane 5: Positive sample for *Eimeria tenella*
Lane 6: Positive sample for *Eimeria acervulina*
Lane 7: Sample for *Eimeria praecox*
Lane 8: Sample for *Eimeria necatrix*
Lane 9: Sample for *Eimeria brunetti*
Lane 10: Sample for *Eimeria maxima*
Lane 11: Negative control

```
Query: Contig1 Query ID: lcl|Query_10713 Length: 728


>Eimeria tenella strain Ingten mitochondrion, complete genome
Sequence ID: KX094951.1 Length: 6213
Range 1: 1640 to 2367

Score:1345 bits(728), Expect:0.0,
Identities:728/728(100%),  Gaps:0/728(0%), Strand: Plus/Plus

Query  1     TAAGTACATCATTAATGTCATTATCTCCAACCTCAGTAGATTTAATTGTATTTGGTTTAG  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1640  TAAGTACATCATTAATGTCATTATCTCCAACCTCAGTAGATTTAATTGTATTTGGTTTAG  1699

Query  61    CTTTATCTGGTATTTCTAGCTTCTTATCATCTATTAATTTCTTAACTACAATTGCTGTAC  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1700  CTTTATCTGGTATTTCTAGCTTCTTATCATCTATTAATTTCTTAACTACAATTGCTGTAC  1759

Query  121   TAGGTGTTACTAATGGTTCAAAACCATGGTGTCTATTTACTTGGGCTATTGTATTCACAG  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1760  TAGGTGTTACTAATGGTTCAAAACCATGGTGTCTATTTACTTGGGCTATTGTATTCACAG  1819

Query  181   CTATTATGTTACTTGGAACACTTCCAATTCTTACAGGTGGATTATTAATGCTGGTACTAG  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1820  CTATTATGTTACTTGGAACACTTCCAATTCTTACAGGTGGATTATTAATGCTGGTACTAG  1879

Query  241   ACTTACATCTAAATACCCAATTCTACGATGCCGCTTTTAATGGTGATCCAGTATTATATC  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1880  ACTTACATCTAAATACCCAATTCTACGATGCCGCTTTTAATGGTGATCCAGTATTATATC  1939

Query  301   AACATCTATTCTGGTTCTTCGGACATCCAGAAGTATATATTATTATTTTACCTGCCTTTG  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1940  AACATCTATTCTGGTTCTTCGGACATCCAGAAGTATATATTATTATTTTACCTGCCTTTG  1999

Query  361   GTGTTGTTTCTCAAACATTATCTACTTCAGCAGGTAAATTAGTATTTGGAGGTCCTTCTA  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2000  GTGTTGTTTCTCAAACATTATCTACTTCAGCAGGTAAATTAGTATTTGGAGGTCCTTCTA  2059

Query  421   TGATCCTTGCTATGGGATGTATTACTGTACTAGGATCATTAGTATGGGCACATCATATGA  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2060  TGATCCTTGCTATGGGATGTATTACTGTACTAGGATCATTAGTATGGGCACATCATATGA  2119

Query  481   TGACAGTTGGTCTAGAAACAGATACTAGAGCATACTTCTCAGCTATTACCATGATGATTG  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2120  TGACAGTTGGTCTAGAAACAGATACTAGAGCATACTTCTCAGCTATTACCATGATGATTG  2179

Query  541   CAATTCCAACAGGTACCAAAATTTTTAACTGGTTAAGTACTTATATGGGAAATCCATTTA  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2180  CAATTCCAACAGGTACCAAAATTTTTAACTGGTTAAGTACTTATATGGGAAATCCATTTA  2239

Query  601   GTACAATATCACTAGATATTTGGTATGCTTTAAGCTTTATTTTCCTATTTACTCTAGGAG  660
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2240  GTACAATATCACTAGATATTTGGTATGCTTTAAGCTTTATTTTCCTATTTACTCTAGGAG  2299

Query  661   GTACCACTGGAGTAGTACTAGGCAATACTGCTTTAGATGTTGCTCTACATGATACATACT  720
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2300  GTACCACTGGAGTAGTACTAGGCAATACTGCTTTAGATGTTGCTCTACATGATACATACT  2359

Query  721   ATGTAATT  728
             ||||||||
Sbjct  2360  ATGTAATT  2367
```

Fig. 4.3: GenBank blast search for *Eimeria spp.* (Universal primer COI gene) haplotype. Query represents haplotype while Sbjct represents *Eimeria tenella.* The query fully aligned (100%) with reference sequence (KX094951)

Table 4.2: Sequences submitted to NCBI database with GenBank Submission ID.

Sr. no.	Geographical origin	Species	Gene(COI/ITS-1) fragment size	% identity	Reference sequence	Country	GenBank Submission ID.
1	Nagpur (MH)	*E. acervulina*	321	99%	AF446056.1	Australia	SUB12864895
2	Nagpur (MH)	*E. tenella* (ITS-1)	278	99%	GQ153633.1	China	SUB12865185
3	Nagpur (MH)	*E. mitis*	306	94%	MT792379.1	South Korea	SUB12865308
4	Nagpur (MH)	*E. tenella* (COI)	810	100%	KX094951.1	Australia	SUB12865254

Alignment of query *Eimeria acervulina* and reference sequence from GenBank; the query aligned 99% with one gap and single mismatch with the reference sequence of *Eimeria acervulina* isolate C internal transcribed spacer 1 (Ref. Seq. ID AF446056.1) shown in (Fig. 4.4).

Alignment of query *Eimeria tenella* (species-specific primer, ITS-1 gene) and reference sequence from GenBank; the query aligned 99% with one gap and one mismatch with the reference sequence of *Eimeria tenella* isolate ETSH4PF3-17 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, complete sequence; and 5.8S ribosomal RNA gene (Ref. Seq. ID GQ153633.1) shown in (Fig. 4.5).

Alignment of query *Eimeria mitis* and reference sequence from GenBank; the query aligned 94% with eight gaps and few mismatches with the reference sequence of *Eimeria mitis* isolate 192-1 internal transcribed spacer one partial sequence (Ref. Seq. ID MT792379.1) shown in (Fig. 4.6).

Isolates of *Eimeria acervulina* and *Eimeria tenella* exhibited 99% identity, while *Eimeria mitis* was 94% with the respective reference sequence. Similar findings were recorded by Lew *et al.* (2003) in Australian isolates of *Eimeria* species.

Phylogenetic distance tree was constructed from highly similar reference sequences for each species presented in (Fig. 4.7, 4.8, 4.9, 4.10).

PCR technique based on the amplification of ITS-1 region of genomic DNA can be efficiently used for *Eimeria* species detection, and in the upcoming era, it will help to develop new and definitive controlling strategies in coccidiosis (Schitzler *et al.,* 1998). Detection by PCR is based on the amplification of the definite region of DNA with the help of specific oligonucleotide primers. However, it requires a high quantity of parasitic material for more effectiveness (Morris & Gasser, 2006). This method selectively amplifies the double-stranded genomic DNA (Siddiki *et al.,* 2014).

4.4 Anticoccidial effects of essential oil of Thymol:

Control of coccidiosis almost depends on sanitation, anticoccidials used in the feeds, dietary status, vaccination, and chemoprophylaxis. It has been investigated that natural feed supplements are helpful in strategies for coccidiosis control as resistance to already existing products is developed (Peek and Landman, 2003). Aromatherapy, i.e., the use of essential oils instead of chemotherapy, is closely related to phytotherapy & it includes the use of distilled plant volatiles. Essential oils differ in chemical composition from other herbal products as the distillation process only recovers lighter phytomolecules (Peek and Landman, 2011). Supplementation of essential oils like thyme and their phenols, such as Thymol and carvacrol, showed a precise reduction in *Eimeria spp.* Oocyst excretion and positive effects on intestinal lesion scores in chicks can be used to prevent & treat coccidiosis in birds (Zhai *et al.,* 2018; Lahlou *et al.,* 2021; Ibrir *et al.,* 2001).

The anticoccidial effect of Thymol has been evaluated by Rondon*et al.* (2006), Felici *et al.* (2020), and Włosek and Swiatkiewicz (2012) with some other essential oils or mixed with herbal extract; this indicated that the effect of Thymol on coccidiosis had been merely studied. Therefore the proposed study has been planned to evaluate the anticoccidial effect of Thymol in comparison to the most widely used ionophores anticoccidial (Maduramycin); the sporulated oocysts of coccidia were sprayed on the litter to study the effects of natural infection.

The OPG count of experimentally infected birds was recorded weekly. Fresh droppings from each group were collected at an interval of 7 days till the end of the trial to count OPG. Droppings from each group were processed, and oocysts were counted 6 times by using McMasters chamber as suggested by (Hodgson, 1970) to record the average OPG value. Results were noted, and statistical analysis was performed with one-way ANOVA (table 4.3). After the 42nd day, three birds from each group were sacrificed with intestinal lesion scoring as per guidelines of Johnson and Reid (1970), and the score was recorded and summarized in table 4.4:

```
Query: 0123-082_007_PCR_A1_EAF_A02 Query ID: lcl|Query_24269 Length: 268
>Eimeria acervulina isolate C internal transcribed spacer 1, complete
sequence
Sequence ID: AF446056.1 Length: 507
Range 1: 167 to 427

Score:470 bits(254), Expect:7e-128,
Identities:259/261(99%),  Gaps:1/261(0%), Strand: Plus/Plus


Query  9    ATTGAA-CTTATCATCTACCAATCTTTGAATCTGTTTTGTTTTCCCACCACGACGCATTT  67
            |||||| |||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  167  ATTGAACCTTATCATCTACCAATCTTTGAATCTGTTTTGTTTTCCCACCACGACGCATTT  226

Query  68   TTGTGAAGAAAAGAAGAGGAAAAAACCTGACTTTGCAAGCATCATTGCCACCTTTTGAAG  127
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  227  TTGTGAAGAAAAGAAGAGGAAAAAACCTGACTTTGCAAGCATCATTGCCACCTTTTGAAG  286

Query  128  GGATGGGATGATGATGCATGCATGGGAgggggaggggcggcgcatgcaccgcttggggct  187
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  287  GGATGGGATGATGATGCATGCATGGGAGGGGGAGGGGCGGCGCATGCACCGCTTGGGGCT  346

Query  188  ttttggggctttggggctgtggtggtggggctTGCATGCTACATGTGACCCTGGCACTGC  247
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  347  TTTTGGGGCTTTGGGGCTGTGGTGGTGGGGCTTGCATGCTACATGTGACCCTGGCACTGC  406

Query  248  TGTCTATGATGAGCACCCATA  268
            ||||||||||||||| |||||
Sbjct  407  TGTCTATGATGAGCATCCATA  427
```

Fig. 4.4: GenBank blast search for *Eimeria acervulina* haplotype. Query represents haplotype while Sbjct represents *Eimeria acervulina.* The query aligned 99% with one gap and single mismatch (showed with arrows) with reference sequence (AF446056)

```
Query: Contig1 Query ID: lcl|Query_35097 Length: 284


>Eimeria tenella isolate ETSH4PF3-17 18S ribosomal RNA gene, partial
sequence; internal transcribed spacer 1, complete sequence; and 5.8S
ribosomal RNA gene, partial sequence
Sequence ID: GQ153633.1 Length: 628
Range 1: 166 to 444

Score:510 bits(276), Expect:4e-140,
Identities:279/280(99%),  Gaps:1/280(0%), Strand: Plus/Minus


Query  3    CGAGCGCTCTGCATACGACACAGCCCCGTGCGCGTCTTCTCCTCTCATAATCCCCTCCAC  62
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  444  CGAGCGCTCTGCATACGACACAGCCCCGTGCGCGTCTTCTCCTCTCATAATCCCCTCCAC  385

Query  63   GTACGTGCGCGATCGACGATCACGGGGCTGGCGCCGGCGCGCTGGGGCTGCCACCGACAG  122
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  384  GTACGTGCGCGATCGACGATCACGGGGCTGGCGCCGGCGCGCTGGGGCTGCCACCGACAG  325

Query  123  GCCCCACGCGAGCCCGTGCATGCGCCACCAcccccccGATCGCCCACGTACATACTACTG  182
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  324  GCCCCACGCGAGCCCGTGCATGCGCCACCACCCCCCCGATCGCCCACGTACATACTACTG  265

Query  183  CTATATATCCTTGCAGCAGCAAAAAATTCCATCCATTTTTAAAAAGTAGAAAAACCGTTG  242
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  264  CTATATATCCTTGCAGCAGCAAAAAATTCCATCCATTTTTAAAAAGTAGAAAAACCGTTG  205

Query  243  CAGAGAAAAACAGATTCAAGGGTTGCGATGGGACTAAATT  282
            ||||||||||||||||||||||||||||||| ||||||||
Sbjct  204  CAGAGAAAAACAGATTCAAGGGTTGCGATGG-ACTAAATT  166
```

Fig. 4.5: GenBank blast search for *Eimeria tenella* haplotype. Query represents haplotype while Sbjct represents *Eimeria tenella*. The query aligned 99% with single gap (showed with arrow) with reference sequence (GQ153633)

```
Query: 0123-400_003_PCR_MT1_EMF_D06.ab1 Query ID: lcl|Query_24365 Length:
277


>Eimeria mitis isolate 192-1 internal transcribed spacer 1, partial
sequence
Sequence ID: MT792379.1 Length: 267
Range 1: 4 to 264

Score:396 bits(214), Expect:1e-105,
Identities:252/269(94%),  Gaps:8/269(2%), Strand: Plus/Plus

Query  3    ATTGGGCTGGCTTTCTGGAACCATTGTTATCCAATCTTTGAATCCATAGTTATTTCTCCA  62
            ||| |||| || |||| |||||||||||||||||||||||||||||||||||||||||||
Sbjct  4    ATT-GGCT-GC-TTCT-GAACCATTGTTATCCAATCTTTGAATCCATAGTTATTTCTCCA  59

Query  63   CGACGTTATTTTTAAAAAGAAGGCTTTTTAAAGTTGTTTTGCCTGCATTTCAACTCGATT  122
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  60   CGACGTTATTTTTAAAAAGAAGGCTTTTTAAAGTTGTTTTGCCTGCATTTCAACTCGATT  119

Query  123  TGCAGGTGTGTGGGATGAGTATGCTTGTTgggggggCGGTCTGTggggggggTTTCTTTG  182
            ||||||||||||||||||||||||||||||||||||  |||  ||||  ||||    |||||
Sbjct  120  TGCAGGTGTGTGGGATGAGTATGCTTGTTGGGGGGTCTGTCGGTGGTGGGG----CTTTG  175

Query  183  GGGCTGCATGTGCATGCATGTATGTGGCTGCACCCCCAAGCTCTGCACACCATGGTGTGT  242
            ||||||||||||||||| || ||||||||||| |||||||||||||||||||||||||||
Sbjct  176  GGGCTGCATGTGCATGCGTGCATGTGGCTGCAGCCCCAAGCTCTGCACACCATGGTGTGT  235

Query  243  TATTACCGGCATCCCGATTCTCGCTTGCA  271
            ||||||||| |||||||||||| ||||||
Sbjct  236  TATTACCGGAATCCCGATTCTCTCTTGCA  264
```

Fig. 4.6: GenBank blast search for *Eimeria mitis* haplotype. Query represents haplotype while Sbjct represents *Eimeria mitis*. The query aligned 94% with eight gaps and few mismatches (showed with arrow) with reference sequence (MT792379)

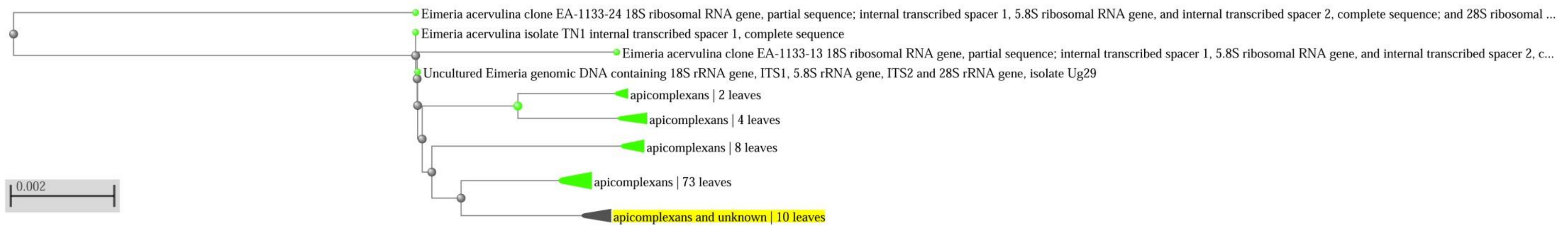

Fig. 4.8: Phylogenetic distance tree of *Eimeria acervulina*

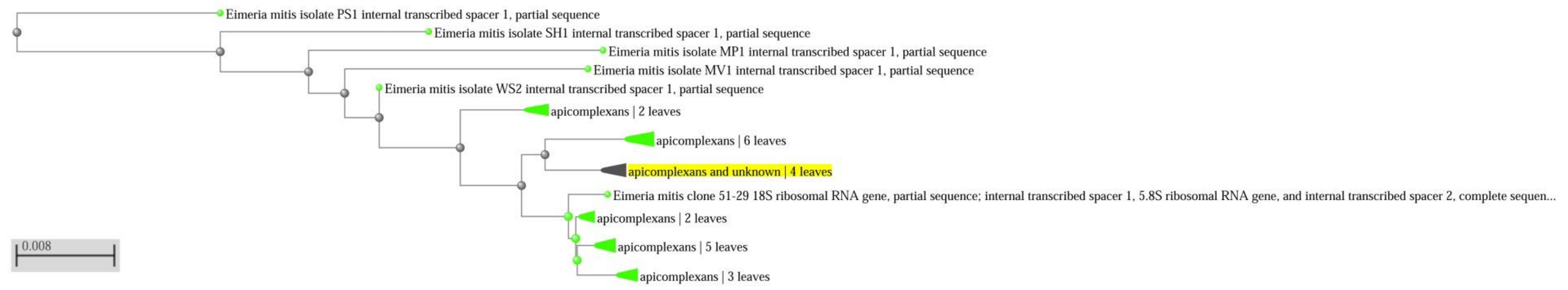

Fig. 4.10: Phylogenetic distance tree of *Eimeria mitis*

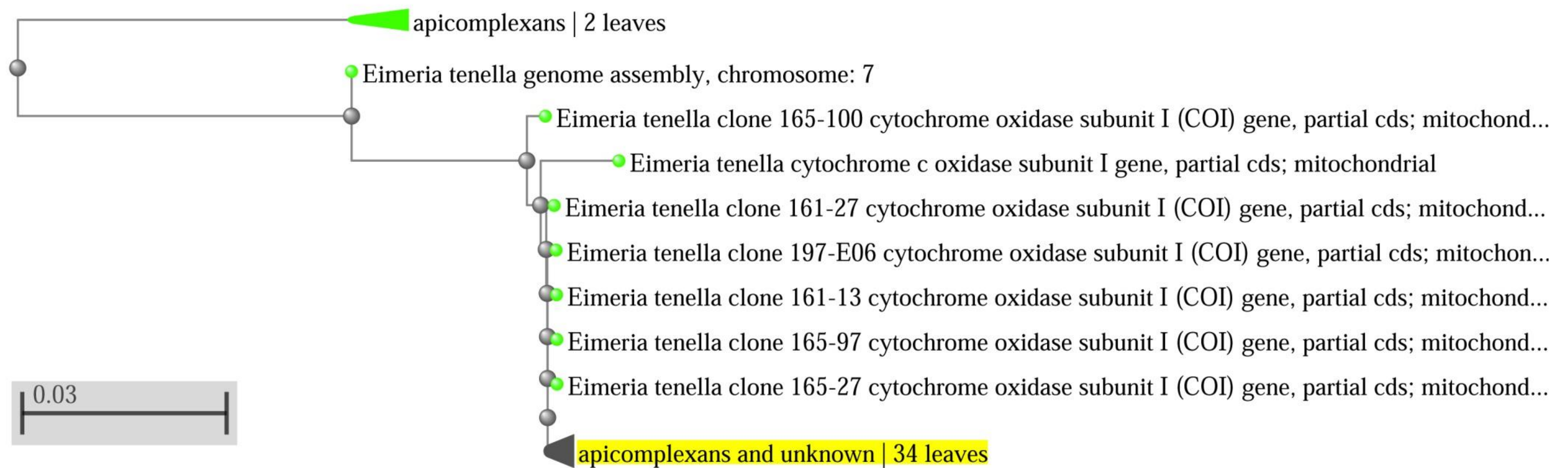

Fig. 4.7: Phylogenetic distance tree of *Eimeria tenella* (COI gene)

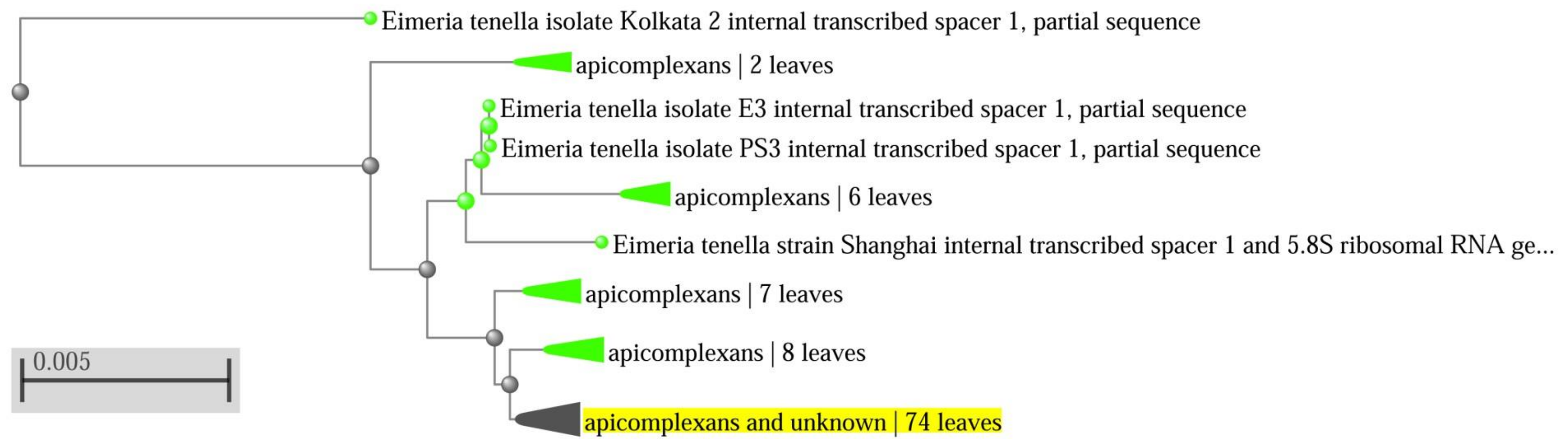

Fig. 4.9: Phylogenetic distance tree of *Eimeria tenella* (ITS-1 gene)

Table 4.3: Results of OPG count:

Groups	Control (A)	Maduramycin (B)	Thymol (C)	SEM	P-value
7th day	0.00	0.00	0.00	0.00	Nil
14th day	166.67[a]	0.00[b]	116.67[a]	24.88	P<0.05
21st day	5650.00[a]	950.00[c]	2100.00[b]	506.11	P<0.001
28th day	45833.33[a]	0.00[b]	50.00[b]	5406.50	P<0.001
35th day	8283.33[a]	0.00[b]	16.67[b]	951.40	P<0.001
42nd day	1300.00[a]	0.00[b]	0.00[b]	160.88	P<0.001

* Means bearing superscripts a, b, and c within rows differ significantly (p≤0.05).

The results of the present experiment indicated that the weekly OPG count in treatment groups differed significantly (P≤0.05) 14th day onwards compared to the control. In the second week, the control and thymol groups recorded significantly (P≤0.05) higher OPG counts than maduramycin. In the third week, OPG was significantly higher (P≤0.001) in the control group, followed by group thymol than maduramycin. During the 4th, 5th & 6th weeks, significantly (P≤0.001) lower OPG equal to nil was observed in both treatment groups over the control group. In the last three weeks, OPG values in the thymol group were significantly lower than in the control, comparable to maduramycin. The trend of decreased OPG count of the thymol essential oil supplementation group was evident till last week as compared to the control group, and nearly equal values were observed as compared to maduramycin. These statistical findings agree with Asli and Rashti (2015); Bozkurt*et al.* (2016).

Table 4.4: Results of lesion scoring:

Group	Duodenum	Mid intestine	Caecum	Rectum	Total
Control (A)	+2,+2,+2	+2,0,+1	+1,+1,+1	0,0,0,	12
Maduramycin (B)	+1,0,0	0,+1,0	0,0,0,	0,0,0	02
Thymol (C)	0,+1,+1	+1,0,0	+1,0,0	0,0,0	04

Polyether Ionophoric compound maduramycin showed the negligible presence of oocysts only on the 21st day of age, and during the entire period, it was null; these results completely agreed with Conway and Mckenzie (2007) that

maduramycin is highly effective against mixed infections of several *Eimeria* species. Its use in poultry as a coccidiostatic feed additive was authorized and approved in 1989 (Anadon and Martinez, 2014). Maduramycin is effective against mild or very severe coccidiosis as compared to other ionophores (McDougald*et al.,* 1987).

In the control group without any treatment, the observed OPG count was very high till the 28th day of age, and there is a periodic increase in this count (Fig: 4.11). This analysis supports, Karaer *et al.* (2012) that age and infection rate are positively co-related. The significantly decreased OPG count in the last two weeks is somewhere related to the development of immunity by birds; similar results were noted by Chapman (1999), wherein he observed that oocyst production in the non-medicated control group was normal till the fourth week. However, significantly low OPG was observed in the last three weeks, which indicated immunity development. He also stated that the treatment of anticoccidials could not be withdrawn despite immunity, as it may decrease production.

This may be because of the continuous exposure of birds to sporulated oocysts since the first day, which provides conditions required for immunity development (Joyner and Norton 1976; Stiff and Bafundo, 1993). Such protective immunity developed by birds is due to the ingestion of a low number of oocysts in the initial stage of life, and after two to three cycles of development of protective immunity leads to solid immunity (Tewari and Maharana 2011). The role of developed immunity or specific antibodies on parasitic development suggested that it may be a new method to control coccidiosis (Lillehoj and Lillehoj 2000).

OPG count for Thymol (Table 4.3) showed a negligible rise up to the 21st day, followed by a gradual decrease, and in the last week, it was null similar to maduramycin. The lesion score for both medicated groups was also nearly similar (Fig: 4.12), which indicated that the essential oil of Thymol could be used as an alternative to existing anticoccidials.

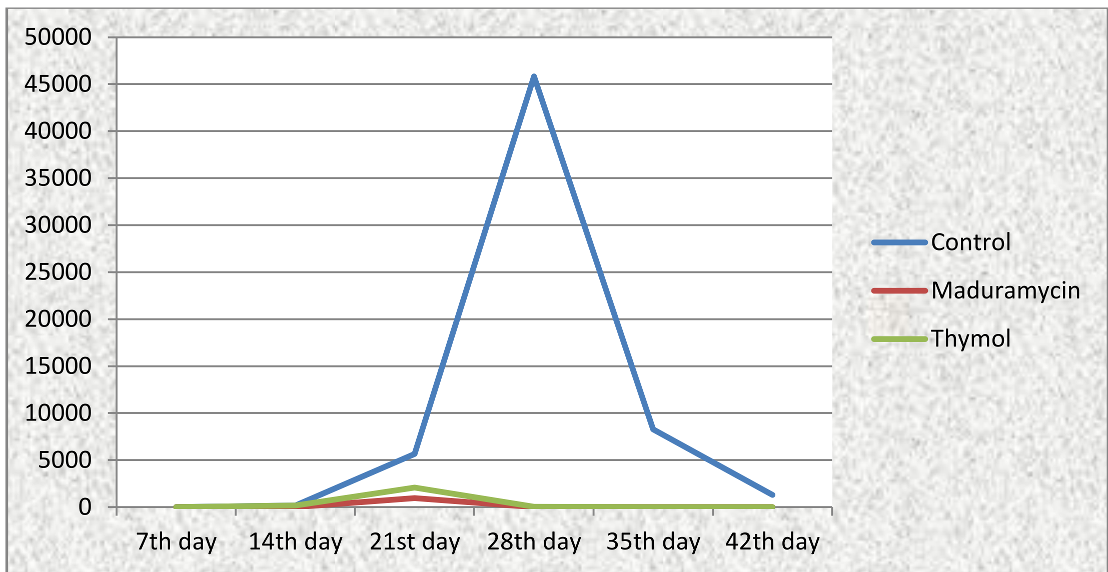

Fig: 4.11 Graph for OPG count

Fig: 4.12 Comparative lesion scoring:

The results recorded in this study are in agreement with Alp *et al.* (2012); Bozkurt *et al.* (2016); Asli and Rashti (2015); Giannenas *et al.* (2003); Tsinas *et al.* (2011), who studied the effects of oregano extract and essential oil as anticoccidial. Tsinas *et al.* (2011) suggested that the actual mechanism of action and significance level of essential oils, when used alone, should be studied. The results are contrary to Włosek and Swiatkiewicz (2012); Scheurer *et al.* (2013) because they used a lesser dosage of the essential oil.

The principal compounds present in oregano essential oil are Thymol, carvacrol, and traces of other monoterpenes (Teixeira *et al.,* 2013). Oregano essential oil and its two major constituents i.e. thymol and carvacrol, impaired cell membranes in micro-organisms (Lambert *et al.,* 2001). Similar results were observed by Rondon *et al.* (2006) against mixed infection of *Eimeria*, but they used EO blends of Thymol, Eugenol, Curcumin, and Piperin.

Remmal *et al.* (2013) & Arafa *et al.* (2020) observed a deformed and ruptured oocyst wall in microscopic studies of oocysts in-vitro treated with Thymol and confirmed its oocysticidal action. On this basis, Arafa *et al.* (2020) also studied the in-vivo effect of Thymol on pigeon coccidiosis and recorded decreased oocyst count and suggested that the effect of Thymol on OPG count is because of monoterpenoid structure and its phenolic properties. The essential oils derived from aromatic plants have phenol as their main chemical compound, which exerts its effects on the host as well as coccidian cells (Tsinas *et al.,* 2011). Naturally derived phenolic compounds are good antioxidants, have positive effects on immunity development, and also they are very good antimicrobials (Mahfuz *et al.,* 2021).

Lesion score for Thymol suggested that dietary use of Thymol helps to maintain intestinal morphology and supported the findings by Yang *et al.* (2018). Clinical coccidiosis is predisposing factor for necrotic enteritis, Williams (2005). The complications and enteric lesions due to *Clostridium perfringes* secondary to coccidiosis infection are found to be reduced when dietary essential oil blend, i.e., Thymol & carvacrol or Thymol, carvacrol & eugenol were used (Hashemipour *et al.,* 2016; Mitsch *et al.,* 2004).

Results of Histopathology:

Tissue samples from the intestine showing gross lesions were collected for histopathology in 10% formalin. After fixation, these tissues were processed using xylene and alcohol followed by impregnation in paraffin wax as per routine method, and 5µ sections were cut and stained with H&E as per described by Luna (1968) for recording histopathologic observations under light microscopy.

Duodenum

Section of the duodenum from the control group (A) revealed severe disruption of villous epithelium, the presence of endogenous developmental stages of coccidian parasite in enterocytes of lamina propria, necrosis of crypts, and inflammatory cells in the submucosa (Plate 4.7). Lymphoid aggregates with shortening of villi and subserosal hemorrhages were also observed in a few sections in group A (Plate 4.8). Similar histopathological lesions were noted in groups B and C, but with less intensity when compared with the control group (Plate 4.9 and 4.10).

Jejunum

Section of jejunum from control group A revealed severe disruption of villous epithelium, the presence of numerous endogenous developmental stages of coccidian parasite in enterocytes of lamina propria, and inflammatory cells in the submucosa (Plate 4.11). Similar histopathological lesions were noted in groups B and C with less intensity when compared with control group A (Plate 4.12 and 4.13).

Ileum

Section of ileum from control group A revealed disintegrated villi and the presence of numerous endogenous developmental stages of the coccidian parasite, i. e. oocysts and macrogametes in enterocytes of lamina propria (Plate 4.14). However, when compared with the control group, mild lesions were observed in groups B and C (Plate 4.15).

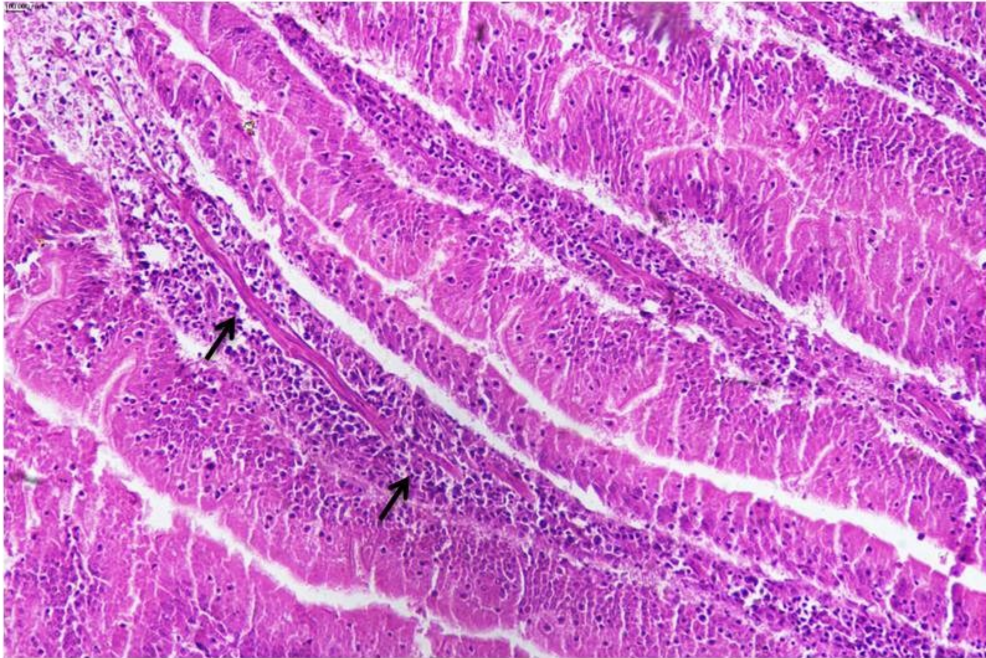

Plate 4.7: Duodenum group A-Numerous endogenous stages of *Eimeria* in enterocytes of villi and cellular infiltration (arrow) H&E, 100x

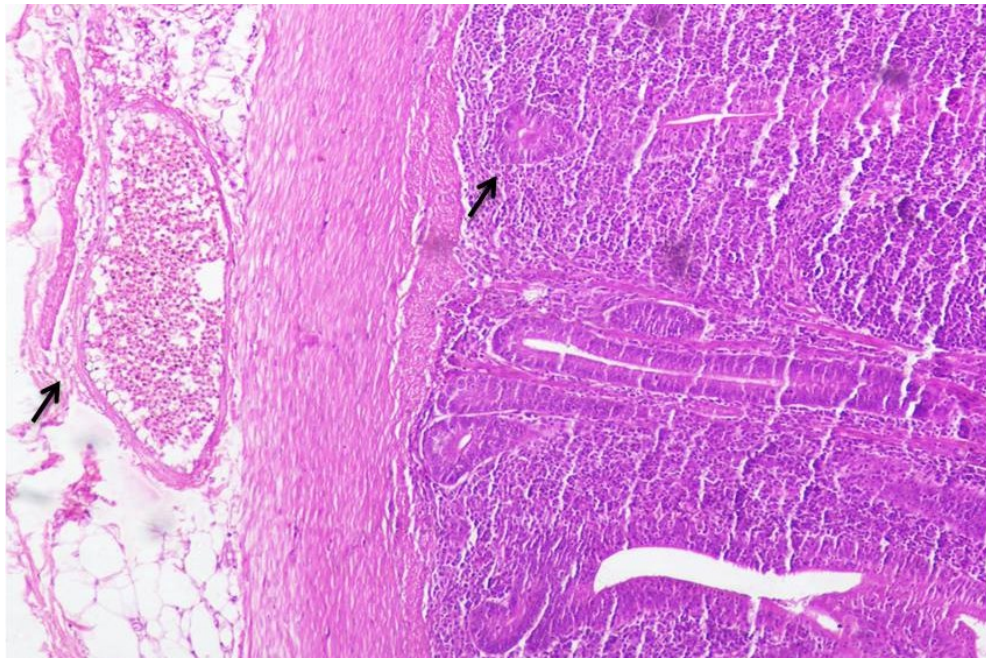

Plate 4.8: Duodenum group A-Lymphoid aggregates with shortening of villi and subserosal haemorrhages H&E, 100x

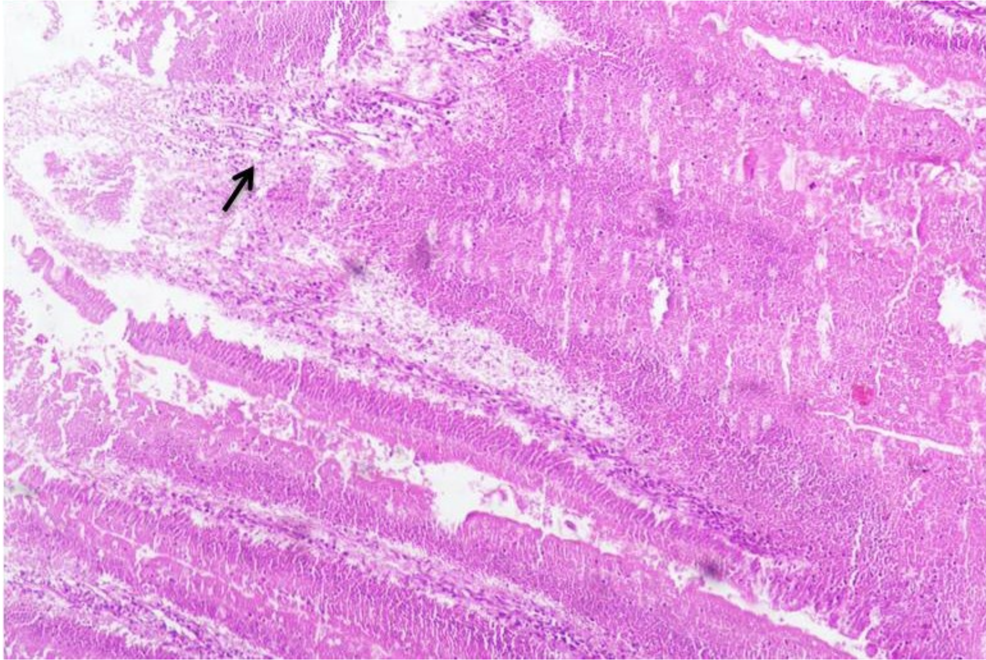

Plate 4.9: Duodenum group B -Crypt necrosis and endogenous stages of *Eimeria* in enterocytes of villi (arrow) H&E, 100x

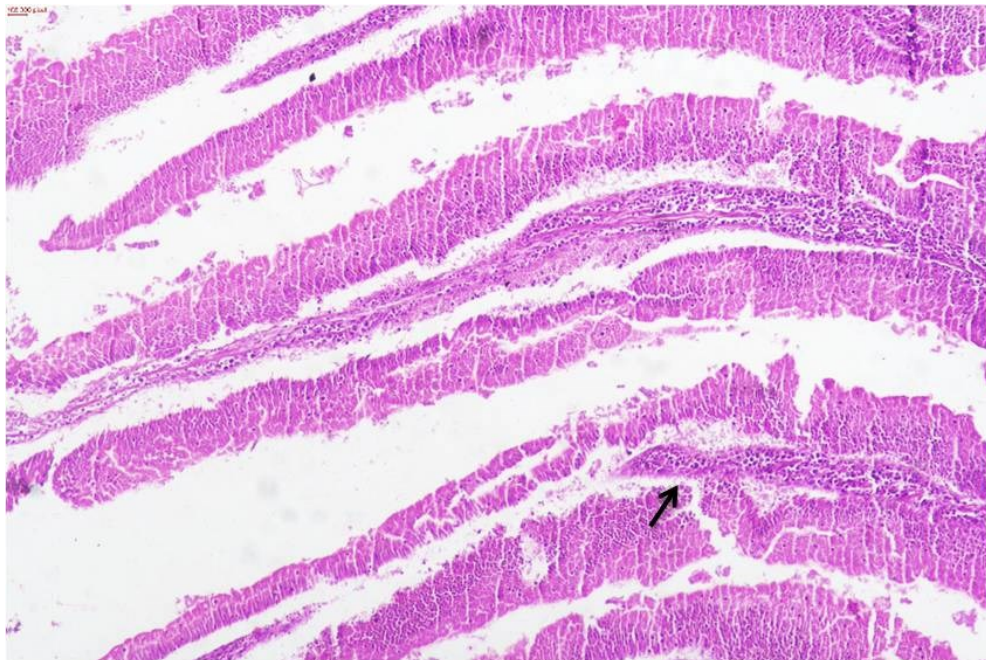

Plate 4.10 : Duodenum group C -Disintegrated villi with few endogenous stages of *Eimeria* in enterocytes, H&E, 100x

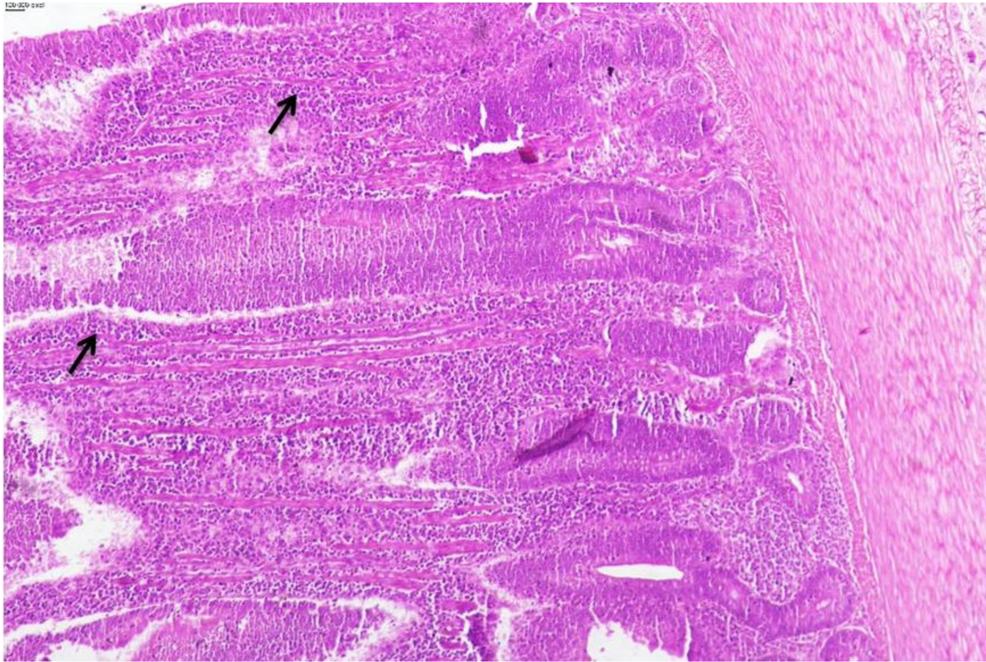

Plate 4.11: Jejunum group A - Numerous endogenous stages of *Eimeria* in enterocytes H&E, 100x

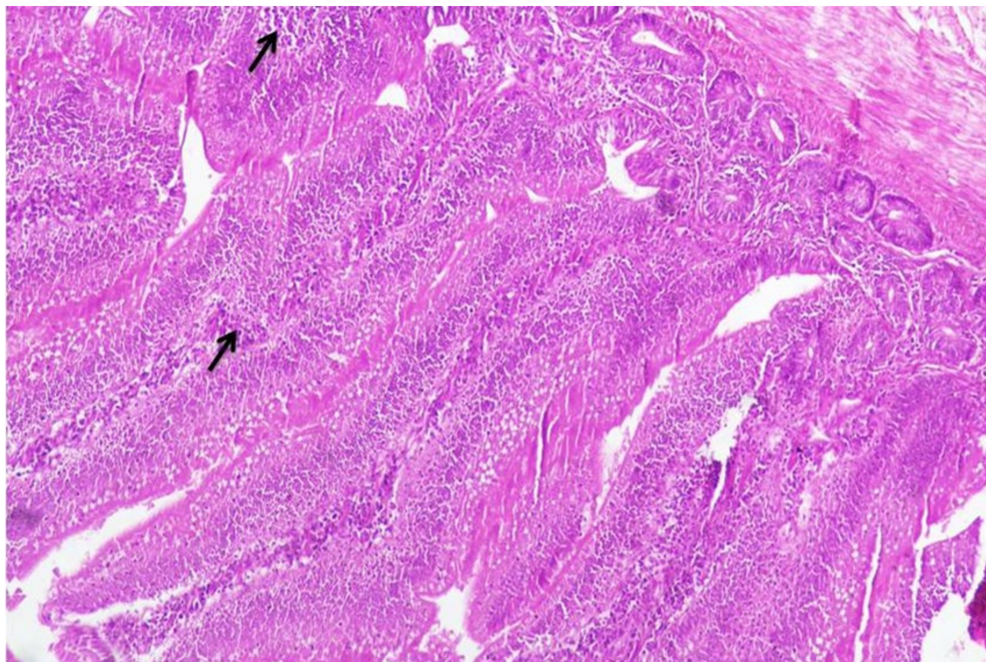

Plate 4.12: Jejunum group B - Few endogenous stages of *Eimeria* in enterocytes H&E, 100x

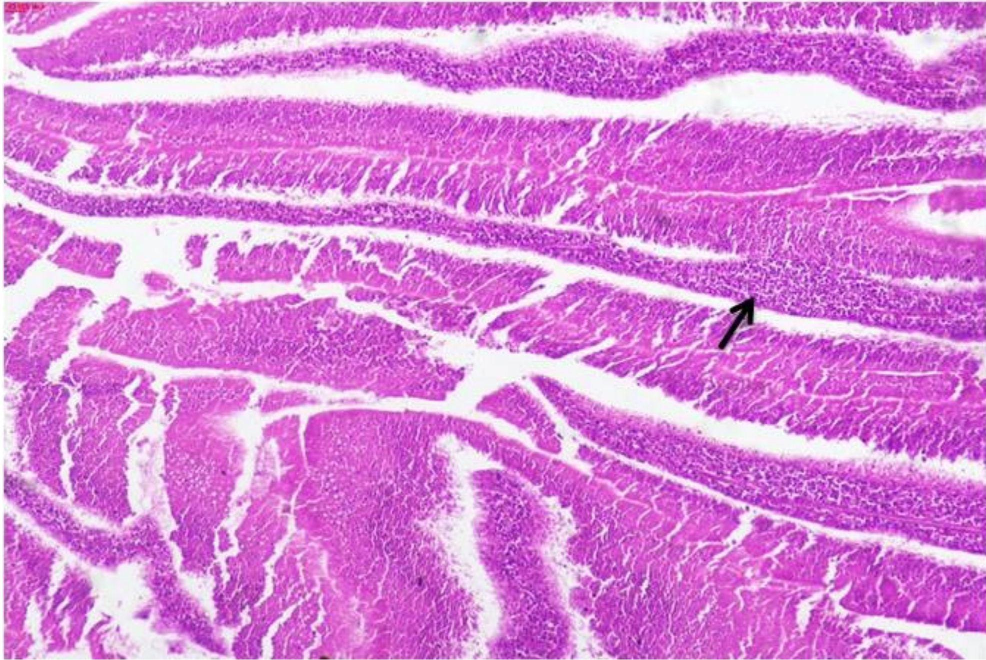

Plate 4.13: Jejunum group C - Few endogenous stages of *Eimeria* in enterocytes H&E, 100x

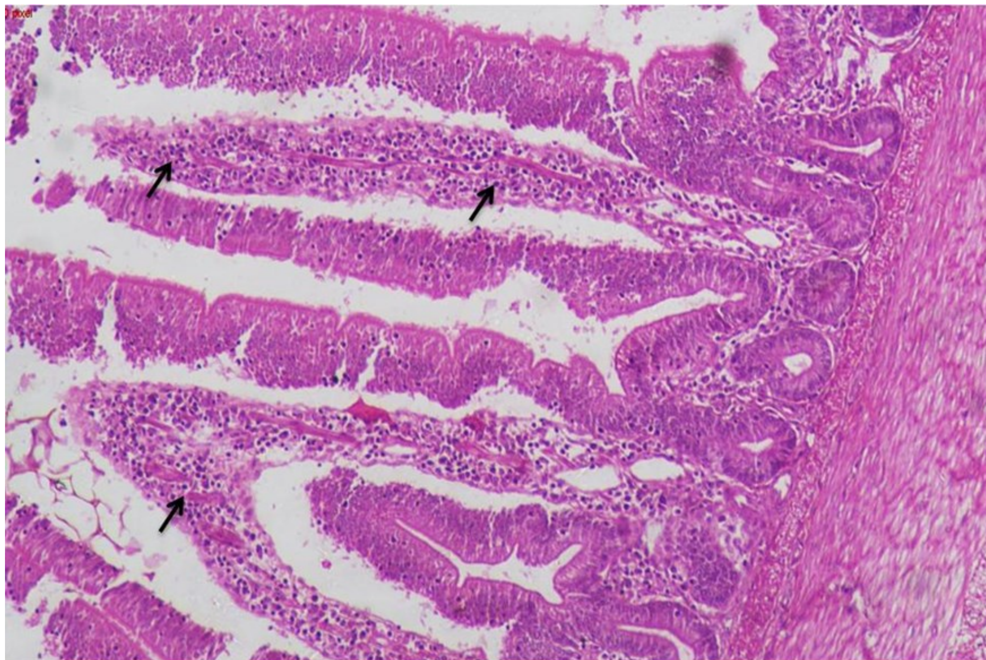

Plate 4.14: Ileum group A - Numerous oocysts having centrally located nucleus and thick wall (arrow) H&E, 100x

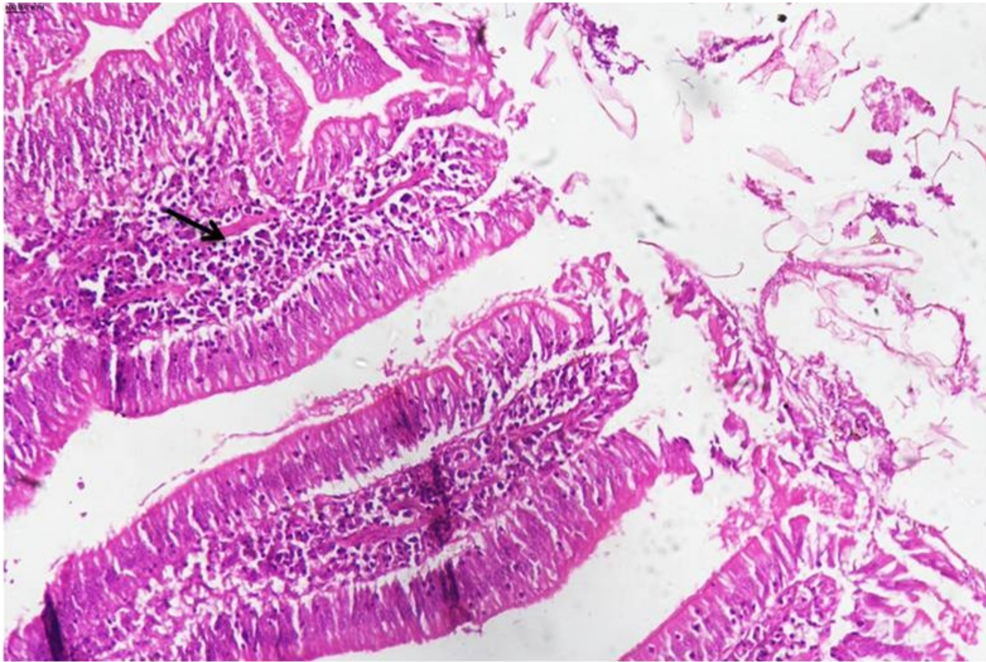

Plate 4.15: Ileum group C - Few endogenous stages of *Eimeria* in enterocytes H&E, 100x

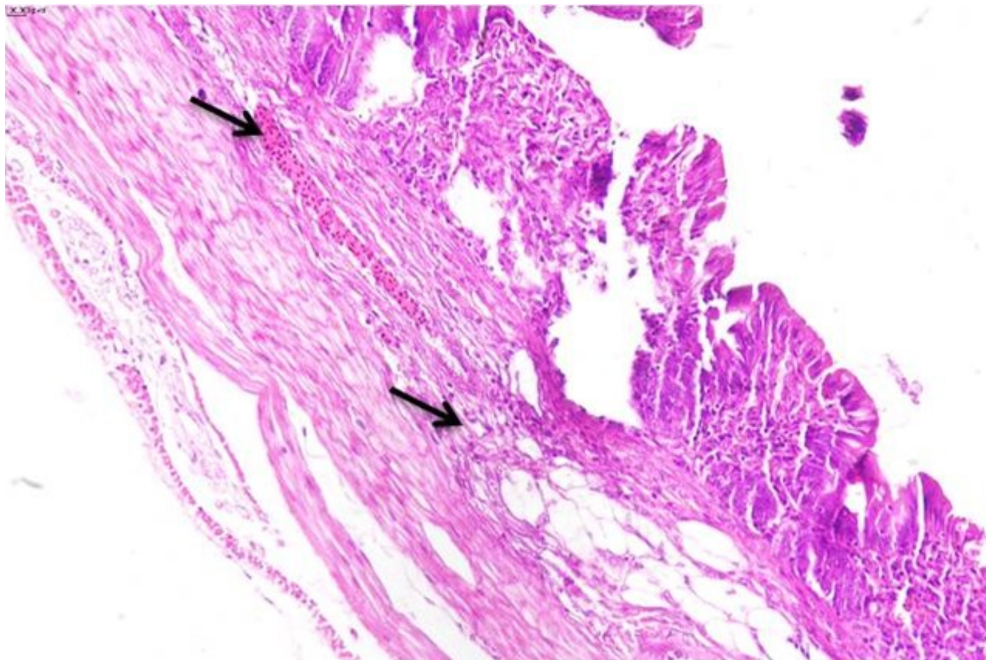

Plate 4.16: Caecum group A-Developmental stages of *Eimeria spp.* in mucosa with disintegrated villi and haemorrhages in the musculareis layer (arrow), H&E, 100x

Caecum

Section of the Caecum from control group A revealed disintegrated villi, hemorrhages in muscularity, and various developmental stages of *Eimeria* in the mucosal layer of the Caecum (Plate 4.16). Few developmental stages of *Eimeria sp*. in the mucosa and mild lesions were observed in groups B and C as compared to the control group (Plate 4.17 and 4.18).

Eimeria infection results in morphologic changes in the intestinal mucosa that reduce the absorptive surface, but it can be reduced by using anticoccidials, Nabian *et al.,* (2018). Above findings suggested that Thymol and maduramycin showed nearly similar effects on developmental stages of *Eimeria* and also decreased the severity of lesions as compared to the control group. Significant results were observed in Thymol treated group in all parts of the intestine. These results are in agreement with Rondon *et al.* (2006), Arafa *et al.* (2020), Asli and Rashti (2015), and Tsinas *et al.* (2011). Contrary to these, Scheurer *et al.* (2013) do not find any effectiveness of herbal blends because of low levels of dosage.

In the present study, it was observed that maduramycin prevented the developmental stages *of Eimeria* and also decreased intestinal lesions. As compared to other ionophoric compounds, maduramycin effectively decreased intestinal lesions in coccidiosis (McDougald *et al.,* 1987). But it is also considered a toxic polyether ionophoric compound, and it has adverse effects on intestinal morphology (Hassanpour *et al.,* 2010). Depending on the severity of the illness, the use of chemical anticoccidials might be either beneficial or detrimental to intestinal morphology, (Nabian *et al.,* 2018). Thymol exerts a protective effect on intestinal mucosa because of its role in cytokine production and anti-inflammatory activity (Ku and Lin 2013) and its destructive effect on developmental stages *Eimeria* (Remmal, *et al.,* 2013). Dietary supplementation of thymol @60mg/ kg decreased the microscopic lesions and improved intestinal histomorphology (Du *et al.,* 2016). Thyme essential oil strengthens intestinal health by altering the permeability and presence of phenolic compounds to prevent oxidative stress at the cellular level (Placha *et al.,* 2014).

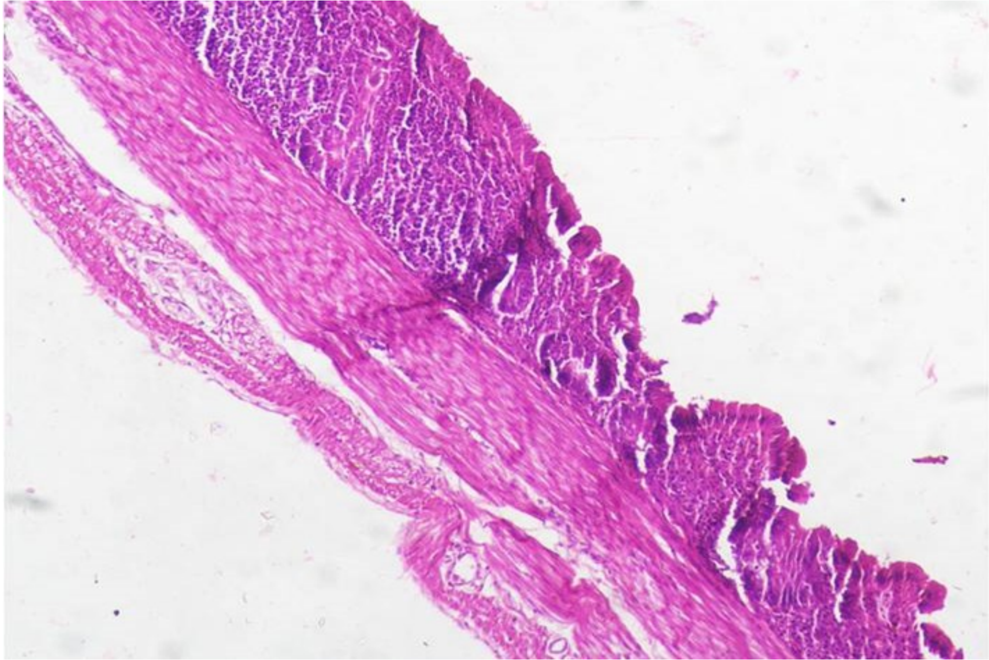

Plate 4.17: Caecum group B–Absence of developmental stages of *Eimeria spp*. in mucosa, H&E, 100x

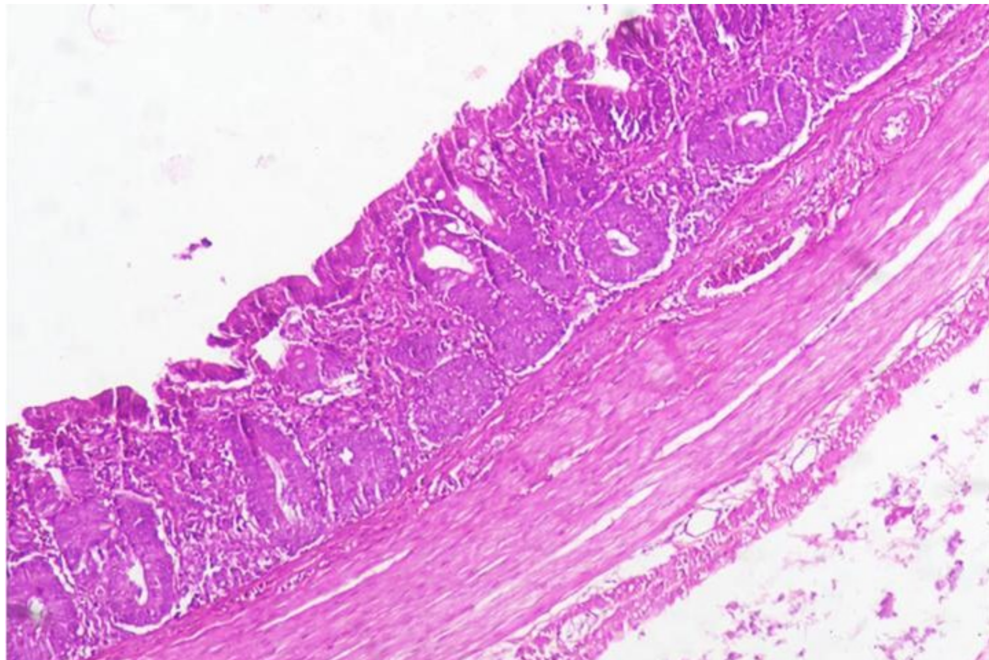

Plate 4.18: Caecum group C –Few developmental stages of *Eimeria spp*. in mucosa, H&E, 100x

SUMMARY AND CONCLUSION

Coccidiosis in poultry is an enteric and intracellular parasitic disease caused by different species of Apicomplexan protozoan parasites of the genus *Eimeria*. Coccidiosis is the most common and most important disease in the economic aspect of poultry worldwide. The accurate diagnosis of coccidiosis in poultry is the primary need for its effective control, and it is important for the investigation of a disease outbreak of coccidiosis. Examination of the drooping sample for the presence of oocyst is the simplest and most accessible diagnostic test for most veterinarians, but it is unreliable for differentiation between species. Molecular methods like PCR gives species level identification, which is further important for the development of vaccines.

In the present study, fecal samples from 30 broiler farms were collected in a plastic container in and around the Nagpur region and brought to the laboratory. Samples were processed by sedimentation technique, and the last drop of sediment was checked under low power of compound microscope (10X) for the presence of *Eimeria* oocysts. Sediments from positive samples were sporulated by using 2.5% potassium dichromate.

By using these sporulated oocysts, a group of broiler birds was challenged for *Eimeria* infection, and in vivo anticoccidial activity of the essential oil of Thymol was checked. Some of the sporulated oocysts were separated by flotation technique for morphometric observations. A polymerase chain Reaction was performed for confirmation of *Eimeria* species, as PCR gives the most sensitive and accurate results.

Out of 30 farms screened, 21 (70%) were found positive for coccidiosis infection, out of which 3 (14.28%) were positive only for *Eimeria tenella*, 11 (52.28%) showed mixed infection with *Eimeria acervulina*, *Eimeria mitis* and *Eimeria tenella* and 7 (33%) showed mixed infection with *Eimeria acervulina* and *Eimeria mitis.*

Microscopic identification based on morphometry & molecular identification by using conventional PCR revealed the presence of only three species, i.e., *Eimeria acervulina*, *Eimeria mitis*, and *Eimeria tenella,* in the Nagpur region.

The anticoccidial effects of the powdered form of Thymol were studied and compared with ionophoric anticoccidial maduramycin. Birds were challenged on day one by spraying *Eimeria* oocysts on the floor. Diets were formulated as per standards excluding coccidiostat, antibiotics, and probiotics to check the effect of Thymol as anticoccidial. The negative control diet contained no coccidiostat or Thymol, whereas the other two diets were supplemented with the powdered form of either Thymol @ 20gms/100kg or a maduramycin @ 50gms / 100kg feed. Fresh droppings from each group were collected at an interval of 7 days till the end of the trial to carry out the OPG count. In the control group, without any treatment, the observed oocyst per gram (OPG) count was very high till the 28th day of age, and there is a periodic increase in this count. Polyether Ionophoric compound maduramycin showed the negligible presence of oocysts only on the 21st day of age, and during the entire period, it was null. OPG count for Thymol showed a negligible rise up to the 21st day, followed by a gradual decrease, and in the last week, it was null similar to maduramycin. No significant difference was found between the activity of Thymol and maduramycin.

CONCLUSION:

Thirty broiler farms were screened for the presence of oocysts of *Eimeria* in and around Nagpur. By molecular technique (PCR), it has been observed that the three species of *Eimeria*, i.e., *Eimeria acervulina*, *Eimeria mitis*, and *Eimeria tenella,* are predominantly found in the Nagpur region.

The experimental infection of coccidia and its treatment with the powdered form of Thymol showed anticoccidial activity at par with the polyether ionophoric anticoccidial maduramycin.

Our experiment concludes that Thymol can be used as an anticoccidial in broiler feed as an alternative to the chemical anticoccidial.

BIBLIOGRAPHY

Aarthi, S., Dhinakar Raj, G., Raman, M., Gomathinayagam, S., & Kumanan, K. (2010). Molecular prevalence and preponderance ofEimeriaspp. among chickens in Tamil Nadu, India. Parasitology Research, 107(4), 1013–1017.

Abbas, R. Z., Iqbal, Z., Blake, D., Khan, M. N., & Saleemi, M. K. (2011). Anticoccidial drug resistance in fowl coccidia: The state of play revisited. World's Poultry Science Journal, 67(2), 337–350.

Abbas, R. Z., Iqbal, Z., Sindhu, Z. -D., Khan, M. N., & Arshad, M. (2008a). Identification of cross resistance and multiple resistance in *Eimeriatenella* field isolates to commonly used anticoccidials in Pakistan. Journal of Applied Poultry Research, 17(3), 361–368. https://doi.org/10.3382/japr.2008-00027

Adams, D. S., Ruiz-Jimenez, F., Fletcher, O. J., Gall, S., & Crespo, R. Image analysis for Eimeriaoocyst counts and classification. (2022). Journal of Applied Poultry Research, 31(3), 100260,ISSN 1056-6171.

Allen, P. C., & Fetterer, R. H. (2002). Recent advances in biology and immunobiology of *Eimeria* species and in diagnosis and control of infection with these coccidian parasites of poultry. Clinical Microbiology Reviews,

Alp, M., Midilli, M., Kocabağlı, N., Yılmaz, H., Turan, N., Gargılı, A., & Acar, N. (2012). The effects of dietary oregano essential oil on live performance, carcass yield, serum immunoglobulin G level, and oocyst count in broilers. Journal of Applied Poultry Research, 21(3), 630–636.

Amer, M. H. H., Awaad, R. M., El-Khateeb, N. M. T. N., Abu-Elezz, A., & Sherein-Said, M. M. (2010). Ghetas and M. A. Kutkat. Isolation and identification of *Eimeria* from field coccidiosis in chickens. Journal of American Science, 6(10), 1107–1114.

Amiri, H. (2012). Essential oils composition and antioxidant properties of three Thymus species. Evidence-Based Complementary and Alternative Medicine: eCAM, 2012, 728065. https://doi.org/10.1155/2012/728065(), 1–8.

Anadón, A., & Martínez-Larrañaga, M. (2014). Veterinary drugs residues: Coccidiostats. Encyclopedia of food safety (pp. 63–75).

Anderson, W. I., Reid, W. M., & Johnson, J. K. (1976). Effects of high environmental temperatures on cecalcoccidiosis. Poultry Science, 55(4),

Anthony, J. P., Fyfe, L., & Smith, H. (2005). Plant active components—A resource for antiparasitic agents? Trends in Parasitology, 21(10), 462–468.

Arafa, W. M., Abolhadid, S. M., Moawad, A., Abdelaty, A. S., Moawad, U. K., Shokier, K. A. M., Shehata, O., & Gadelhaq, S. M. (2020). Thymol efficacy against coccidiosis in pigeon (Columba liviadomestica). Preventive Veterinary Medicine, 176, 104914.

Asli , M., &Ghanaatparast-Rashti, M. (2015). Dietary oregano essential oil alleviates experimentally induced coccidiosis in broilers. Preventive Veterinary Medicine, 120(2), 195–202. https://doi.org/10.1016/j.prevetmed.2015.03.014

Awais, M. M., Akhtar, M., Iqbal, Z., Muhammad, F., & Anwar, M. I. (2012). Seasonal prevalence of coccidiosis in industrial broiler chickens in Faisalabad, Punjab, Pakistan. Tropical Animal Health and Production, 44(2), 323–328. https://doi.org/10.1007/s11250-011-0024-x

Barbour, E. K., Bragg, R. R., Karrouf, G., Iyer, A., Azhar, E., Harakeh, S., &Kumosani, T. (2015). Control of eight predominant Eimeria spp. involved in economic coccidiosis of broiler chicken by a chemically characterized essential oil. Journal of Applied Microbiology, 118(3), 583–591. https://doi.org/10.1111/jam.12731

Barta JR, Martin DS, Liberator PAet al. (1998) Phylogenetic relationships among eight Eimeriaspecies infecting domestic fowl inferred using complete small subunit ribosomal DNA sequences.Journal ofParasitology83: 262–271

Barta,JR (2001). Coccidiosis. Encyclopedia of Life Sciences. doi:10.1038/npg.els.0001947

Barta, J. R., Martin, D. S., Liberator, P. A., Dashkevicz, M., Anderson, J. W., Feighner, S. D., Elbrecht, A., Perkins-Barrow, A., Jenkins, M. C., Danforth, H. D., Ruff, M. D., & Profous-Juchelka, H. (1997). Phylogenetic relationships among eight *Eimeria* species infecting domestic fowl inferred using complete small subunit ribosomal DNA sequences. Journal of Parasitology, 83(2), 262–271. https://doi.org/10.2307/3284453

Bawer, M. D., Gs, M., Puttalakshamamma, G., Ananda, K., Lakkundi, J. N., & R, S. (2021). Comparison of different DNA extraction procedures for *Eimeria* oocysts. Journal of Entomology and Zoology Studies, 9(2), 1010–1014. https://doi.org/10.22271/j.ento.2021.v9.i2l.8568

Becker, E. R. (1959). Protozoa. In Biester & Schwarte (Eds.), Diseases of poultry (4th ed) (pp. 828–916), Chapter 36.

Becker. (1948). Chap. 35. Diseases of poultry. In H. E. Biester & L. H. Schwarte (Eds.), Protozoa (2nd ed) (pp. 863–946). Iowa State College Press.

Bera, A. K., Bhattacharya, D., Pan, D., Dhara, A., Kumar, S., & Das, S. K. (2010). Evaluation of economic losses due to coccidiosis in poultry industry in India. Agricultural Economics Research Review, 23, 91–96.

Bhaskaran, M. S., Venkatesan, L., Aadimoolam, R., TirunelveliJayagopal, H., & Sriraman, R. (2010) Sequence diversity of internal transcribed spacer-1 (ITS-1) region of Eimeria infecting chicken and its relevance in species

identification from Indian field samples. Parasitology Research, 106(2), 513–521. https://doi.org/10.1007/s00436-009-1696-2

Bozkurt, M., Ege, G., Aysul, N., Akşit, H., Tüzün, A. E., Küçükyılmaz, K., Borum, A. E., Uygun, M., Akşit, D., Aypak, S., Şimşek, E., Seyrek, K., Koçer, B., Bintaş, E., & Orojpour, A. (2016). Effect of anticoccidial monensin with oregano essential oil on broilers experimentally challenged with mixed Eimeria spp. Poultry Science, 95(8), 1858–1868.

Brown Jordan, A., Blake, D., Beard, J., Beharry, A., Serrette, L., Soleyn, A., Sookhoo, J., Blake, L., Brown, G., &Oura, C. (2018). Molecular identification of *Eimeria* species in broiler chickens in Trinidad, West Indies. Veterinary Sciences, 5(1), 12–. https://doi.org/10.3390/vetsci5010012

Carvalho, F. S., Wenceslau, A. A., Teixeira, M., Matos Carneiro, J. A., Melo, A. D. B., & Albuquerque, G. R. (2011). Diagnosis of *Eimeria* species using traditional and molecular methods in field studies. Veterinary Parasitology, 176(2–3), 95–100. https://doi.org/10.1016/j.vetpar.2010.11.015

Cha, J. O., Talha, A. F., Lim, C. W., & Kim, B. (2014, March). Effects of glass bead size, vortexing speed and duration on *Eimeria Acervulina* oocyst excystation. Experimental Parasitology, 138, 18–24. Epub January 20, 2014. PubMed: 24457170.

Chapman, H. D. (1997). Biochemical, genetic and applied aspects of drug resistance in *Eimeria* parasites of the fowl. Avian Pathology, 26(2), 221–244.

Chapman, H. D. (1999). The development of immunity to *Eimeria* species in broilers given anticoccidial drugs. Avian Pathology, 28(2), 155–162.

Chapman, H. D., Cherry, T. E., Danforth, H. D., Richards, G., Shirley, M. W., & Williams, R. B. (2002). Sustainable coccidiosis control in poultry production: The role of live vaccines. International Journal for Parasitology, 32(5), 617–

Chere, M. A., Melese, K., &Megerssa, Y. C. (2022). Molecular characterization of *Eimeria* species in broiler chickens, Ethiopia. Veterinary Medicine, 13, 153–161. https://doi.org/10.2147/VMRR.S357432, PubMed: 35945979, PubMed Central: PMC9357388

Clark, E. L., & Blake, D. P. (2012). Genetic mapping and coccidial parasites: Past achievements and future prospects. Journal of Biosciences, 37(5), 879–886. https://doi.org/10.1007/s12038-012-9251-1

Conway, D. P., & McKenzie, M. E. (2007). Poultry coccidiosis: Diagnostic and testing procedures. Wiley-Blackwell.

Conway, D. P., McKenzie, M. E., & Dayton, A. D. (1990). Relationship of coccidial lesion scores and weight gain ininfections of *Eimeria acervulina*, *E. maxima*, and *E. Tenella* in broilers. Avian Pathology, 19(3), 489–496.

Costa, C. A., Gomes, R. F., Melo, M. N., &Ribeiro, M. F. (2001). Eimeriaparasites of domestic fowl: Genetic relationships of different isolates estimated from random amplified polymorphic DNA. Parasitology Research, 87(6), 459–466.

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

Dalloul, R. A., &Lillehoj, H. S. (2006). Poultry coccidiosis: Recent advancements in control measures and vaccine development. Expert Review of Vaccines, 5(1), 143–163.

Daszak, P. (1999). Zoite migration during *Eimeria tenella* infection: Parasite adaptation to host defences. Parasitology Today, 15(2), 67–72.

Du, E., Wang, W., Gan, L., Li, Z., Guo, S., &Guo, Y. (2016). Effects of thymol and carvacrol supplementation on intestinal integrity and immune responses of

broiler chickens challenged with Clostridium perfringens. Journal of Animal Science and Biotechnology, 7(1), 19. https://doi.org/10.1186/s40104-016-0079-7

Duszynski, D. W., & Wilber, P. G. (1997). A guideline for the preparation of species descriptions in the Eimeriidae. Journal of Parasitology, 83(2), 333–336. https://doi.org/10.2307/3284470

Edgar, S. A., &Seibold, C. T. (1964). A New Coccidium of Chickens, *Eimeria mivati* sp. n. (Protozoa: Eimeriidae) with Details of Its Life History. Journal of Parasitology, 50(2), 193–204. https://doi.org/10.2307/3276267

Fanatico, A. (2006). Parasite management for natural and organic poultry Coccidiosis. http://attra. ncat. Org./attar- pub/PDF/coccidiosis.pdf. www. Saxonet. De/coccido2.htm.

Felici, M., Tugnoli, B., Ghiselli, F., Massi, P., Tosi, G., Fiorentini, L., Piva, A., & Grilli, E. (2020): https:. In vitro anticoccidial activity of thymol, carvacrol, and saponins. Poultry Science, 99(11), 5350–5355. https://doi.org/10.1016/j.psj.2020.07.035

Geng, T., & ChengYeC. Y, ZhenyuLei, BangShen. S, RuiFang, MinHuM.H.;, JunlongZhao;, YanqinZhouY. Z.;, Fang, R.;, Hu, M.;, Zhao, J.;, Zhou, Y., . . . Zhou, Y.; (2021). Prevalence of *Eimeria* parasites in the Hubei and Henan provinces of China. Parasitology Research, 120(2), 655–663.

Gharekhani, J., Sadeghi-Dehkordi, Z., &Bahrami, M. (2014). Prevalence of coccidiosis in broiler chicken farms in Western Iran. Journal of Veterinary Medicine, 2014, 980604.

Giannenas, I., Florou-Paneri, P., Papazahariadou, M., Christaki, E., Botsoglou, N. A., & Spais, A. B. (2003). Effect of dietary supplementation with oregano

essential oil on performance of broilers after experimental infection with *Eimeria tenella*. Archiv Fur Tierernahrung, 57(2), 99–106.

Györke, A., Pop, L., & Cozma, V. (2013). Prevalence and distribution of *Eimeria* species in broiler chicken farms of different capacities. Parasite, 20. https://doi.org/10.1051/parasite/2013052(), 50–. doi:10.1051/parasite/2013052

Harper, G. C., & Makatouni, A. (2002). Consumer perception of organic food production and farm animal welfare. British Food Journal, 104(3/4/5), 287–299. https://doi.org/10.1108/00070700210425723

Hashemipour, H., Khaksar, V., Rubio, L. A., Veldkamp, T., & van Krimpen, M. M. (2016). Effect of feed supplementation with a thymol plus carvacrol mixture, in combination or not with an NSP-degrading enzyme, on productive and physiological parameters of broilers fed on wheat-based diets. Animal Feed Science and Technology, 211, 117–131.

Hassanpour, H., Bahadoran, S., Koosha, S., Askari, E., &Homai, S. (2010). Effect of diclazuril, semduramicin, salinomycin and maduramycin as preventive anticoccidial drugs on chicken intestinal morphology. Global Veterinaria, 5, 1–5.

Haug, A., Gjevre, A. G., Thebo, P., Mattsson, J. G., &Kaldhusdal, M. (2008). Coccidial infections in commercial broilers: Epidemiological aspects and comparison of *Eimeria* species identification by morphometric and polymerase chain reaction techniques. Avian Pathology, 37(2), 161–170. https://doi.org/10.1080/03079450801915130

Hill, N. J., Richter, C., & Power, M. L. Pinning down a polymorphic parasite: New genetic and morphological descriptions of *Eimeria* macropodis from the

tammar wallaby (Macropuseugenii). (2012). Parasitology International, 61(3), 461–465. https://doi.org/10.1016/j.parint.2012.03.003

Hodgson, J. N. (1970). Coccidiosis: Oocyst counting technique for coccidiostat evaluation. Experimental Parasitology, 28(1), 99–102. https://doi.org/10.1016/0014-4894(70)90073-1

Ibrir, F., Greathead, H. M. R., & Forbes, J. M. (2001). The effect of thymol/carvacrol treatments on the performance of broiler chickens infected with *Eimeria* acervulina. Procedure alternative feed antibiotics anticoccidials in the pig and Poultry. Meat production. Oslo.

Innes, E. A., &Vermeulen, A. N. (2006). Vaccination as a control strategy against the coccidial parasites *Eimeria*, Toxoplasma and Neospora. Parasitology, 133(Suppl.), S145–S168. https://doi.org/10.1017/S0031182006001855

IQBAL, M. & Islam, Jamal, Alam, Masud, Begum, &Shahana. (2017). Identification of existing coccidian species in broiler chickens of Bangladesh. Research Journal of Animal, Veterinary and Fishery Sciences. Md and Bhuiyan, 5, 01–04.

Iqbal, M., & Begum, S. (2018). Prevalence of coccidiosis in broiler chicken.

Jadhav, B. N., & Nikam, S. V. (2014). Study of seasonal incidence of chicken coccidiosis in Gangapur and Vaijapur tehsil of Aurangabad district in Maharashtra. International Journal of Applied Science – Research and Review, 1(3), 93–97.

Johnson, J., & Malcolm Reid, W. (1970). Anticoccidial drugs: Lesion scoring techniques in battery and floor-pen experiments with chickens, 28(1), 0–36. https://doi.org/10.1016/0014-4894(70)90063-9

Jones, C. T., Hunt, D. R., & King, W. N. (1996).Veterinary Ppathology (6th ed.) (pp. 552). USAnitedStates:.Lippincott Williams and Wilkings

Jordan, F., Pattison, M., Alexander, D., & Faragher, T. (2002). Parasitic diseases. In Poultry Disease (5thed.) (pp. 405-420). Hong Kong: W.B. Saunders

Joyner, L. P., & Long, P. L. (1974). The specific characters of the Eimeria, with special reference to the coccidia of the fowl. Avian Pathology, 3(3), 145–157. https://doi.org/10.1080/03079457409353827

Joyner, L. P., & Norton, C. C. (1976). The immunity arising from continuous low-level infection with *Eimeria maxima* and *Eimeria Acervulina*. Parasitology, 72(1), 115–125. https://doi.org/10.1017/s0031182000043250

Kala, S., Gattani, A., Kumar, A., & Samantaray, S. (2013). Infectious dynamics of different species of *Eimeria* in chicken. Animal Science Reporter, 7, 139–145.

Kalita, A., Kakati, P., &Sarmah, P. C. (2021). Isolation and molecular identification of *Eimeria* species circulating in broiler chicken flocks of Assam (India). Haryana Vet., 60(2), 251–254.

Kant P., Verma, Pawan, Bais, Isha, Parmar, Mehtab, Gopal, Anu, Gupta, & Vijayta. (2013). Kant, Vinay and Singh. Anticoccidial Drugs Used in the Poultry, 1, 261–265. https://doi.org/10.5567/sciintl.2013.261.265

Karaer, Z., Guven, E., Akcay, A., Kar, S., Nalbantoglu, S., & Cakmak, A. (2012). Prevalence of subclinical coccidiosis in broiler farms in Turkey. Tropical Animal Health and Production, 44(3), 589–594. https://doi.org/10.1007/s11250-011-9940-z

Kawahara Fumiya, K., Kensuke Taira, K., Shinya Nagai, S., & Hiroshi Onaga, H. (2008). MisaoOnuma and, & Tetsuo Nunoya, Tetsuo. detection of five avian

Eimeria species by species-specific real-time polymerase chain reaction assay. Avian Diseases, 52(4), 652–656. https://doi.org/10.2307/27565809

Kawazoe, U., & Di Fabio, J. D. (1994). Resistance to diclazuril in field isolates of *Eimeria* species obtained from commercial broiler flocks in Brazil. Avian Pathology, 23(2), 305–311. https://doi.org/10.1080/03079459408418998

Ku Chi Me , Jin-YuarnLini. (2013). , anti-inflammatory effects of 27 selected terpenoid compounds tested through modulating Th1/Th2 cytokine secretion profiles using murine primary splenocytes, Food Chemistry, 141(2), 1104–1113, ISSN 0308-8146. https://doi.org/10.1016/j.foodchem.2013.04.044

Kucera, J. (1990). Identification of *Eimeria* species in Czechoslovakia. Avian Pathology, 19(1), 59–66. https://doi.org/10.1080/03079459008418656

Kumar, S., Garg, R., Banerjee, P. S., Ram, H., Kundu, K., Kumar, S., &Mandal, M. (2015). Genetic diversity within ITS-1 region of *Eimeria* species infecting chickens of north India. Infection, Genetics and Evolution, 36, 262–267.

Kumar, S., Garg, R., Moftah, A., Clark, E. L., Macdonald, S. E., Chaudhry, A. S., Sparagano, O., Banerjee, P. S., Kundu, K., Tomley, F. M., & Blake, D. P. (2014). An optimised protocol for molecular identification of *Eimeria* from chickens. Veterinary Parasitology, 199(1–2), 24–31.

Kumar, S., Garg, R., Ram, H., Maurya, P. S., & Banerjee, P. S. (2015). Gastrointestinal parasitic infections in chickens of upper gangetic plains of India with special reference to poultry coccidiosis. Journal of Parasitic Diseases, 39(1), 22–26.

Kumar, S., Stecher, G., & Tamura, K. (2016). MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Molecular Biology Evolution version 7.0, 33(7), 1870–1874.. Epub March 22, 2016. PubMed: 27004904, PubMed Central: PMC8210823

Lahlou, M.B., ArezkiMohammedi, L. R., Silva, G. A., Silva, L. R., Alves, G., . . . Alves, G. (2021). Dietary use of Rosmarinus officinalis and Thymus vulgaris as anticoccidial alternatives in poultry. Animal Feed Science and Technology, 273.

Lambert, R. J., Skandamis, P. N., Coote, P. J., & Nychas, G. J. (2001, September). A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. Journal of Applied Microbiology, 91(3), 453–462.

Lawal, J. R., Jajere, S. M., Ibrahim, U. I., Geidam, Y. A., Gulani, I. A., Musa, G., &Ibekwe, B. U. (2016). Prevalence of coccidiosis among village and exotic breed of chickens in Maiduguri, Nigeria. Veterinary World, 9(6), 653–659. https://doi.org/10.14202/vetworld.2016.653-659

Lee, B. H., Kim, W. H., Jeong, J., Yoo, J., Kwon, Y.-K., Jung, B. Y., Kwon, J. H., Lillehoj, H. S. MIN, & Min, W. (2010). Prevalence and cross-immunity of eimeria species on Korean chicken farms. Journal of Veterinary Medical Science, 72(8), 985–989. https://doi.org/10.1292/jvms.09-0517

Levine, N. D. (1982). Taxonomy and life cycles of coccidia. In P. L. Long (Ed.), The biology of the coccidia (pp. 1–33). University Park Press.

Lew, A. E., Anderson, G. R., Minchin, C. M., Jeston, P. J., & Jorgensen, W. K. (2003). Inter- and intra-strain variation and PCR detection of the internal transcribed spacer 1 (ITS-1) sequences of Australian isolates of Eimeria species from chickens. Veterinary Parasitology, 112(1–2), 33–50.

Lillehoj, H. S., &Lillehoj, E. P. (2000). Avian coccidiosis. A review of acquired intestinal immunity and vaccination strategies. Avian Diseases, 44(2), 408–425. https://doi.org/10.2307/1592556

Long, P. L., & Horton-Smith, C. (1968). Coccidia and coccidiosis in the domestic fowl. Advances in Parasitology, 313–325.

Long, P. L., & Joyner, L. P. (1984). Problems in the identification of species of *Eimeria.* Journal of Protozoology, 31(4), 535–541.

Long, P. L., & Millard, B. J. (1979a). Rejection of Eimeria by foreign hosts. Parasitology, 78(2), 239–247.

Luna, L. G. (1968). Manual of histologic staining methods of the Armed Forces Institute of Pathology (3rd ed). McGraw-Hill.

Lundén, A., Thebo, P., Gunnarsson, S., Hooshmian-Rad, P., Tauson, R., & Uggla, A. (2000). *Eimeria* infections in litter-based, high stocking density systems for loose-housed laying hens in Sweden. British Poultry Science, 41(4), 440–447.

Luu, L., Bettridge, J., & Christley, R. & Melese, K. & Blake, DamerandDessie, Tadelle & Wigley, Paul & Desta, Takele & Hanotte, Olivier & Kaiser, Pete &Terfa, Zelalem& Collins, Marisol & Lynch, Stacey. (2013). Prevalence and molecular characterisation of *Eimeria* species in Ethiopian village chickens. BMC veterinary research. 9. 208. 10.1186/1746-6148-9-208.

Mahfuz, S., Shang, Q., & Piao, Xiangshu. (2021). Phenolic compounds as natural feed additives in poultry and swine diets: A review. Journal of Animal Science and Biotechnology, 12(1), 48. https://doi.org/10.1186/s40104-021-00565-3, (), –. doi:10.1186/s40104-021-00565-3

Matsubayashi, M., Shibahara, T., Matsuo, T., Hatabu, T., Yamagishi, J., Sasai, K., &Isobe, T. (2020). Morphological and molecular identification of *Eimeria* spp. in breeding chicken farms of Japan. Journal of Veterinary Medical Science, 82(5), 516–519. https://doi.org/10.1292/jvms.19-0661, (), –. doi:10.1292/jvms.19-0661

Mcallister, C. T., & Upton, S. J. (1989). The coccidia (Apicomplexa: Eimeriidae) of testudines, with descriptions of three new species. Canadian Journal of Zoology, 67(10), 2459–2467. https://doi.org/10.1139/z89-347

McDonald, V., & Shirley, M. W. (2009) Past and future: Vaccination against *Eimeria.* Parasitology, 136(12), 1477–1489.

McDougald, L. R., Wang, G. T., Kantor, S., Schenkel, R., & Quarles, C. (1987). Efficacy of maduramicin against ionophore-tolerant field isolates of coccidia in broilers. Avian Diseases, 31(2), 302–308. https://doi.org/10.2307/1590876

McManus, E. C., Campbell, W. C., &Cuckler, A. C. (1968). Development of resistance to quinolinecoccidiostats under field and laboratory conditions. Journal of Parasitology, 54(6), 1190–1193. https://doi.org/10.2307/3276989

Mitsch, P., Zitterl-Eglseer, K., Köhler, B., Gabler, C., Losa, R., &Zimpernik, I. (2004). The effect of two different blends of essential oil components on the proliferation of Clostridium perfringens in the intestines of broiler chickens. Poultry Science, 83(4), 669–675. https://doi.org/10.1093/ps/83.4.669

Mohammad A. Q., Suleiman, M. M., & Abo-Shehada, M. N. (2002). Flock-level prevalence of *Eimeria* species among broiler chicks in northern Jordan. Preventive Veterinary Medicine, 53(4), 305–310.

Molloy, J. B., Eaves, F. W., Jeston, P. J., Minchin, C. M., Stewart, N. P., Lew, A. E., & Jorgensen, W. K. (1998). Detection of *Eimeria acervulina* using the polymerase chain reaction. Avian Diseases, 42(1), 119–123.

Moore, E. N., & Brown, J. A. (1951). A new coccidium pathogenic for turkeys, *Eimeria adenoeides* N. SP. (Protozoa: Eimeriidae). Cornell Veterinarian, 41(2), 124–135. PubMed: 14840008

Morris, G. M., & Gasser, R. B. (2006). Biotechnological advances in the diagnosis of avian coccidiosis and the analysis of genetic variation in *Eimeria.* Biotechnology Advances, 24(6), 590–603.

Muazu, A., Masdooq, A. A., Ngbede, J., Salihu, A. E., Haruna, G., Habu, A. K., Sati, M. N., &Jamilu, H. (2008). Prevalence and identification of species of *Eimeria* causing coccidiosis in Poultry with in Vom, Plateau State, Nigeria. International Journal of Poultry Science, 7(9), 917–918.

Musa, I. W., Sa'idu, L., Jatau, J. T., Adamu, J., Otu, M. O., & Abdu, P. A. (2010). Outbreak of coccidiosis in 5-day old commercial breeder flock IN Zaria, Nigeria. International Journal of Poultry Science, 9(12), 1112–1115., Google Scholar [Ref list]

Nabian, S., Arabkhazaeli, F., Seifouri, P., & Farahani, A. (2018, July–September). Morphometric analysis of the intestine in experimental coccidiosis in broilers treated with anticoccidial drugs. Iranian Journal of Parasitology, 13(3), 493–499. PubMed: 30483343, PubMed Central: PMC6243159

Nikam, S. V., Jadhav, B. N., &Bhamre, S. N. (2012). Study of genus *Eimeria* (*Eimeria mitis*, Tyzzer 1929) in broiler chickens (Gallus gallusdomesticus) from Aurangabad (M.S) India. Trends Parasitol. Res., 1(1), 39–41.

Norton, C. C., & Chard, M. J. (1983). The oocyst sporulation time of *Eimeria* species from the fowl. Parasitology, 86(2), 193–198.

Nour, H., HamzeSuleimanMohamoudCisman, M., Ahmed, Z. A., Mohamoud, H. A., TahirAwale, A., Suleiman, H., &Nour, H. (2020). Scope specification of coccidiosis in the poultry on researchers. International Journal of Avian & Wildlife Biology international Journal of Avian and Wildlife Biology, 5(2), 32—37.

Oljira, D., Melaku, A., & A. (2012),. “Prevalence and risk factors of coccidiosis in poultry farms in and around Ambo Town, western Ethiopia. The American-Eurasian Journal of Science Research,. andand B. Bogale, B., 7(4), 146–149.

Pant, S., Bhatt, P., Shekhar, S., & Krishna, G. (2018). Epidemiological investigation of poultry coccidiosis in and around Tarai region of Uttarakhand. International Journal of Current Microbiology and Applied Sciences, 7(7), 374–380. Parasitol. Int.

Parker, B. B., & Duszynski, D. W. (1986). Coccidiosis of sandhill cranes (Gruscanadensis) wintering in New Mexico. Journal of Wildlife Diseases, 22(1), 25–35.

Patterson, F. D. (1933–). Cross infection experiments with coccidia of birds. Cornell Veterinarian, 23, 249–253.

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

Peek, H. W., & Landman, W. J. M. (2011). Coccidiosis in poultry: Anticoccidial products, vaccines and other prevention strategies. Veterinary Quarterly, 31(3), 143–161.

Persia, M. E., Young, E. L., Utterback, P. L., & Parsons, C. M. (2006). Effects of dietary ingredients and *Eimeria acervulina* infection on chick performance, apparent metabolizable energy, and amino acid digestibility. Poultry Science, 85(1), 48–55.

Placha, I., Takacova, J., Ryzner, M., Cobanova, K., Laukova, A., Strompfova, V., Venglovska, K., &Faix, S. (2014). Effect of thyme essential oil and selenium on intestine integrity and antioxidant status of broilers. British Poultry Science, 55(1), 105–114.

Prakashbabu Chengat, B., Thenmozhi, V., Limon, G., Kundu, K., Kumar, S., Garg, R., Clark, E. L., SrinivasaRao, A. S. R., Raj, D. G., Raman, M., Banerjee, P. S., Tomley, F. M., Guitian, J., & Blake, D. P. (2017). Eimeria species occurrence varies between geographic regions and poultry production systems and may influence parasite genetic diversity. Veterinary Parasitology, 233, 62–72.

Qi, N., Liao, S., Abuzeid, A. M. I., Li, J., Wu, C., Lv, M., Lin, X., Hu, J., Xiao, W., Sun, M., & Li, G. (2020). Effect of different floatation solutions on *E. tenella* oocyst purification and optimization of centrifugation conditions for improved recovery of oocysts and sporocysts. Experimental Parasitology, 217, 107965. Epub August 18, 2020. PubMed: 32818513

Quiroz-Castañeda, R. E., & Dantán-González, E. (2015). Control of avian coccidiosis: Future and present natural alternatives. BioMed Research International, 2015, 430610. https://doi.org/10.1155/2015/430610. Epub February 17, 2015. PubMed: 25785269, PubMed Central: PMC4346696

Rao, V. P, Raman, M., & Raj, D. G, Abdul Basith.S and S.Gomathinayagam. (2012). Speciation of Poultry *Eimeria* by morphology and SCAR PCR in Southern India. Indian Journal of Animal Sciences. Indian Journal of Animal Sciences. 82, 82(8), 805–811, 805–811.

Remmal, A., Achahbar, S., Bouddine, L., Chami, F., &Chami, N. (2013). Oocysticidal effect of essential oil components against chicken *Eimeria* oocysts. International Journal of Veterinary Medicine: Research and Reports, 1–8. https://doi.org/10.5171/2013.599816

Remmal, A., Achahbar, S., Bouddine, L., Chami, N., &Chami, F. (2011) In vitro destruction of *Eimeria* oocysts by essential oils. Veterinary Parasitology, 182(2–4), 121–126. https://doi.org/10.1016/j.vetpar.2011.06.002

Rodon et al., (2006). Essential oils on mixed coccidia vaccination and infection in broilers. International Journal of Poultry Sci.ence, 5(8):, 723–730. https://doi.org/10.3923/ijps.2006.723.730

Ruff, M. D., Chute, M. B., &Mcloughlin, D. K. (1985). Resistance to monensin medication in *Eimeria tenella*. Proceedings of the Helminthology Society of Washington, 52, 114–118.

Scheurer, W., Spring, P., &Maertens, L. (2013). Effect of 3 dietary phytogenic products on production performance and coccidiosis in challenged broiler chickens. Journal of Applied Poultry Research, 22(3), 591–599. https://doi.org/10.3382/japr.2013-00726

Schnitzler, B. E., Thebo, P. L., Mattsson, J. G., Tomley, F. M., & Shirley, M. W. (1998). Development of a diagnostic PCR assay for the detection and discrimination of four pathogenic (i) species of the chicken. Avian Pathology, 27(5), 490–497. https://doi.org/10.1080/03079459808419373

Schnitzler, B. E., Thebo, P. L., Tomley, F. M., Uggla, A., & Shirley, M. W. (1999). PCR identification of chicken *Eimeria*: A simplified read-out. Avian Pathology, 28(1), 89–93. https://doi.org/10.1080/03079459995091

Schwarz, R. S., Jenkins, M. C., Klopp, S., &Miska, K. B. (2009). Genomic analysis of Eimeria spp. populations in relation to performance levels of broiler chicken farms in Arkansas and North Carolina. Journal of Parasitology, 95(4), 871–880. https://doi.org/10.1645/GE-1898.1, PubMed: 20049993

Sharma, S., Iqbal, A., Azmi, S., & Shah, H. A. (2013). Study of poultry coccidiosis in organized and backyard farms of Jammu region. Veterinary World, 6(8), 467–469. https://doi.org/10.5455/vetworld.2013.467-469

Shirley, M. W., & Harvey, D. A. (2000). A genetic linkage map of the apicomplexan protozoan parasite eimeriatenella. Genome Research, 10(10), 1587–1593. https://doi.org/10.1101/gr.149200

Shirley, M. W., Smith, A. L., Tomley, F. M., Tomley, F. M. (2005). The biology of avian Eimeria with an emphasis on their control by vaccination. Advances in Parasitology, 60, 285–330. https://doi.org/10.1016/S0065-308X(05)60005-The Biology of Avian *Eimeria* with an Emphasis on their Control by Vaccination. 285–330. doi:10.1016/s0065-308x(05)60005-x

Shirzad, M. R., Seifi, S., Gheisari, H. R., &Abdi, B. Hachesoo; HasanHabibi; Hossein Bujmehrani (2011). Prevalence and risk factors for subclinical coccidiosis in broiler chicken farms in Mazandaran Province, Iran, 43(8), 1601–1604. https://doi.org/10.1007/s11250-011-9876-3

Siddiki, A. Z., Mina, S.A., Anayet Hasan, M., Touaha Akbar, M., Alam, R., Ashraful Islam, M., Zahan Ira, I., & Ayesa, B. (2014). Molecular characterization of *Eimeria* spp. from chicken by Polymerase Chain Reaction based on species-specific SCAR markers, Md& Akbar, Mohammed &Alam, Rashel& Islam, Md& Ira, Israt&Ayesa, Bibi. IOSR Journal of Agriculture and Veterinary Science, 7(1), 13–17.

Singla, L. D., S. K., & Gupta. (2013). Advances in diagnosis of coccidiosis. In poultry

Soulsby, E. J. L. (1982). Helminths, arthropods and protozoan's of domesticated animals (7th ed) p. 630. Bailliere Tindall.

Stamatakis, A. (2014). RAxML version 8: A tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics, 30(9, May), 1312–1313.

Stephen, B., Rommel, M., Daugschies, A., & Haberkorn, A. (1997). Studies of resistance to anticoccidials in *Eimeria* field isolates and pure *Eimeria* strains.

Veterinary Parasitology, 69(1–2), 19–29. https://doi.org/10.1016/s0304-4017(96)01096-5

Stiff, M. I., & Bafundo, K. W. (1993). Development of Immunity in Broilers Continuously Exposed to *Eimeria* sp. Avian Diseases, 37(2), 295–301. https://doi.org/10.2307/1591652

Sun, X. M., Pang, W., Jia, T., Yan, W. C., He, G., Hao, L. L., Bentué, M., &Suo, X. (2009). Prevalence of *Eimeria* species in broilers with subclinical signs from fifty farms. Avian Diseases Digest, 4(2), e28–e28.

Teixeira, B., Marques, A., Ramos, C., Serrano, C., Matos, O., Neng, N. R., Nogueira, J. M. F., Saraiva, J. A., &Nunes, M. L. (2013). Chemical composition and bioactivity of different oregano (Origanum vulgare) extracts and essential oil. Journal of the Science of Food and Agriculture, 93(11), 2707–2714.

Tewari, A. K., &Maharana, B. R. (2011). Control of poultry coccidiosis: Changing trends. Journal of Parasitic Diseases, 35(1), 10–17. https://doi.org/10.1007/s12639-011-0034-7

Thebo, P., Lundén, A., Uggla, A., & Hooshmand-Rad, P. (1998). Identification of seven *Eimeria* species in Swedish domestic fowl. Avian Pathology, 27(6), 613–617.

Tsinas, A., Giannenas, I., Voidarou, C., Tzora, A., & Skoufos, J. (2011). Effects of an oregano based dietary supplement on performance of broiler chickens experimentally infected with *Eimeria acervulina* and *Eimeria maxima*. Journal of Poultry Science, 48(3), 194–200. https://doi.org/10.2141/jpsa.010123

Tyzzer, E. E. (1929a). Methods for isolating and differentiating species of Eimeria occurring in gallinaceous birds. Journal of Parasitology, 15, 148–149.

Tyzzer, E. E. 1929b. Coccidiosis in gallinaceous birds. American Journal of Hygiene, 10, 269–383.M.M.

Vrba, V., & Pakandl, M. (2015). Host specificity of turkey and chicken Eimeria: Controlled cross-transmission studies and a phylogenetic view. Veterinary Parasitology, 208(3–4), (118–124).

Williams, R. B. (2002). Anticoccidial vaccines for broiler chickens: Pathways to success. Avian Pathology, 31(4), 317–353.

Williams, R. B. (2005). Inter current coccidiosis and necrotic enteritis of chickens: Rational, integrated disease management by maintenance of gut integrity. Avian Pathology, 34(3), 159–180.

Włosek, A., & Swi. (2012). Ąt kiewicz, S. The effect of a dietary herbal extract blend on the performance of broilers challenged with *Eimeria* oocysts. Journal of Animal and Feed Sciences, 21, 133–142.

Woods, W. G., Richards, G., Whithear, K. G., Anderson, G. R., Jorgensen, W. K., & Gasser, R. B. (2000). High-resolution electrophoretic procedures for the identification of fiveEimeria species from chickens, and detection of population variation. Electrophoresis, 21(17), 3558–3563.

Yadav, A., & Gupta, S. K. (2001). Study of resistance against some ionophores in *Eimeria tenella* field isolates. Veterinary Parasitology, 102(1–2), 69–75.

Yang, X., Long, F., Xin, H., Yang, C., & Yang, X. (2018). Impact of essential oils and organic acids on the growth performance, digestive functions and immunity of broiler chickens. Animal Nutrition, 4(4), 388–393.

Zhai, H., Liu, H., Wang, S., Wu, J., & Kluenter, A. M. (2018). Potential of essential oils for poultry and pigs. Animal Nutrition, 4(2), 179–186.

APPENDIX

Reagents for Polymerase Chain Reaction (PCR):

1. Reagents for agarose gel electrophoresis:

Tris base/Tris buffer	292 gm
Glacial acetic acid	57.1 ml
0.5 M EDTA (PH 8.0)	100 ml
Distilled water	Upto1000 ml

For working solution, dilute 1:50 (1X TAE) for agarose gel electrophoresis:

50XTAE buffer	20 ml
Distilled water	980 ml
Total Volume	1000 ml
Store at room temperature	

2. Gel loading dye:

Blue/Orange 6X loading dye	1µl
100 bp DNA ladder	5µl
Total volume	6µl
Store at -20˚C	

3. Ethidium bromide solution (10mg/ml):

Ethidium bromide	0.1 gm
Distilled water	10ml
Store at 4˚C	

VITA

The author Dr. Pranav Pralhadrao Deshmukh is born on 16th November, 1997 at Parbhani district of Maharashtra. He has completed his S.S.C. in the year 2013, from Gandhi Vidyalaya, Parbhani and H.S.C. from Yeshwant Mahavidyalaya, Nanded. The author has completed his B.V.Sc & A.H. degree course from Nagpur Veterinary College, Nagpur in the year 2020. He joined Department of Veterinary Parasitology, Nagpur Veterinary College, Nagpur for post graduation studies during 2021-2023.

THESIS ABSTRACT

a)	Title of the thesis	:	**MORPHOLOGICAL AND MOLECULAR IDENTIFICATION OF *EIMERIA SPP*. OF BROILER CHICKENS IN NAGPUR REGION (MAHARASHTRA STATE)**
b)	Full name of student	:	**DESHMUKH PRANAV PRALHADRAO**
c)	Name and address of Major Advisor	:	(Dr. S. G. Jadhao) Assistant Professor Department of Veterinary Parasitology Nagpur Veterinary College, Nagpur
d)	Degree to be awarded	:	**Master of Veterinary Science**
e)	Year of award of degree	:	**2023**
f)	Major subject	:	**Veterinary Parasitology**
g)	Total number of pages in the thesis	:	**49**
h)	Number of words in the abstract	:	**299**
i)	Signature of Student	:	
j)	Signature, Name and address of forwarding authority		

Associate Dean
Nagpur Veterinary College,
Nagpur

ABSTRACT

Coccidiosis in poultry is an enteric and intracellular parasitic disease caused by different species of protozoan parasites of the genus *Eimeria.* Coccidiosis is the most common and most important disease in the economic aspect of poultry worldwide. An incidence of coccidiosis in poultry in India, as

well as in foreign countries, has been widely reported. Detection and diagnosis of *Eimeria* species infections are mostly made through microscopic examination of the faecal sample and molecular and serological techniques. Faecal sample examination based on the presence of oocyst is the most straightforward and accessible diagnostic test for most veterinarians, but it is unreliable for differentiation between species.

The main goal of the current study was to identify the species of *Eimeria* that are present in the Nagpur region using morphometry and the conventional PCR method, as well as to examine the anticoccidial effects of Thymol essential oil on a group of broilers that were coccidiosis-challenged. Faecal samples from 30 broiler farms in and around the Nagpur region were collected for the current investigation and transported to the laboratory in a plastic container.

Using sporulated oocysts, a group of broiler birds was challenged for *Eimeria* infection, and in-vivo anticoccidial activity of the essential oil of Thymol was checked. Some of the sporulated oocysts were separated by flotation technique for morphometric observations. For the most sensitive and precise results, a polymerase chain reaction (PCR) was used to validate the *Eimeria* species.

Out of 30 farms screened, 21 (70%) were found positive for coccidiosis infection, out of which 3 (14.28%) were positive only for *Eimeria tenella*, 11 (52.28%) showed mixed infection with *Eimeria acervulina*, *Eimeria mitis* and *Eimeria tenella* and 7 (33%) showed mixed infection with *Eimeria acervulina* and *Eimeria mitis.*

Only three species of *Eimeria*, namely *Eimeria acervulina, Eimeria mitis*, and *Eimeria tenella*, were found in the Nagpur region after microscopic identification based on morphometry and molecular identification using conventional PCR.

Compared to the ionophoric anticoccidial maduramycin, the anticoccidial effects of thymol powder were examined. When compared to the polyether ionophoric anticoccidial maduramycin, Thymol exhibited comparable anticoccidial action. According to the results of our investigation, Thymol can be replaced chemical anticoccidials in broiler feed.

प्रबंध सारांश

अ.	प्रबंधाचे शिर्षक	:	नागपूर विभागातील (महाराष्ट्र राज्य) मांसल कोंबड्यांमधील आयमेरिया प्रजातींची मॉर्फोमेट्री आणि आण्विक पद्धतीव्दारे ओळख.
ब.	विद्यार्थ्याचे पुर्ण नांव	:	प्रणव प्रल्हादराव देशमुख
क.	मार्गदर्शकाचे नांव व पत्ता	:	डॉ. सुरेश गुलाबराव जाधव सहायक प्राध्यापक पशुवैदयकीय परजीवीशास्त्र विभाग, नागपूर पशुवैदयकीय महाविद्यालय, नागपूर.
ड.	प्रदान करण्यात येणारी पदवी	:	**स्नातकोत्तर पदवी (एम. व्ही. एससी.)**
इ.	पदवी प्रदान करण्याचे वर्ष	:	२०२३
फ	मुख्य विषय	:	पशुवैद्यकीय परजीवशास्त्र
ग.	प्रबंधातील एकूण पृष्ट	:	४९
ह.	सारांशातील एकूण शब्द	:	२९०
ई.	विद्यार्थ्याची सही	:	
		:	
ज.	अग्रेषित करणा-या अधिका-याची सही, नांव आणी पत्ता		सहयोगी अधिष्ठाता नागपूर पशूवैद्यकीय महाविद्यालय नागपूर

<u>सारांश</u>

कोंबड्यांमधील रक्तीहगवण हा एक आंतरीक आणि अंतःकोशिकीय परजीवी रोग आहे जो आयमेरिया वंशाच्या प्रोटोझोआन परजीवींच्या विविध प्रजातींमुळे होतो.

कोक्सीडिओसिस हा जगभरातील कुक्कुटपालनातील सर्वात सामान्य आणि आर्थिक बाबतीत सर्वात महत्वाचा रोग आहे. भारतातील तसेच परदेशात मांसलपक्ष्यामधील कोक्सीडिओसिसचे प्रमाण मोठ्या प्रमाणावर नोंदवल्या गेलेआहे. आयमेरिया प्रजातींच्या संसर्गाचे निदान हे मुख्यतः मायक्रोस्कोपच्या साहाय्याने विष्ठा नमुनातपासणी तसेच आण्विक आणि सेरोलॉजिकल तंत्रांच्याद्वारे केले जाते. उसिस्ट च्या उपस्थितीवर आधारित विष्ठा नमुना तपासणी ही बहुतेक पशुवैद्यकांसाठी सर्वात सोपी निदान चाचणी आहे. परंतु प्रजातींमधील फरकासाठी ती पुर्णपणे विश्वसनीय नाही.

सदरसंशोधनाचे मुख्य उद्दिष्ट हे मॉर्फोमेट्री आणि पीसीआर पद्धतीचा वापर करून नागपूर विभागात आढळणाऱ्या आयमेरियाच्या विविध प्रजाती ओळखणे तसेच कोक्सीडिओसिस प्रादुर्भावित केलेल्या मांसल पक्ष्यांच्या गटावर थायमॉलच्या प्रभावांचे निरीक्षण करणे हे होते. सध्याच्या तपासणीसाठी नागपूर आणि आजूबाजूच्या ३० ब्रॉयलर फार्ममधील पक्ष्यांचे विष्ठा नमुने गोळा करण्यात आले आणि प्लास्टिकच्या डबीमध्ये ते प्रयोगशाळेत पाठवण्यात आले.

स्पोर्युलेटेड उसिस्ट वापरून ब्रॉयलर पक्ष्यांच्या गटाला आयमेरिया संसर्गासाठी आव्हान दिले गेले आणि त्यावर औषधी म्हणून थायमॉलचा प्रभाव तपासण्यात आला. काही स्पोर्युलेटेड उसिस्ट मॉर्फोमेट्रिक निरीक्षणासाठी फ्लोटेशन तंत्राने वेगळे केले गेले. अत्यंत संवेदनशील आणि अचूक परिणामांसाठी तसेच आयमेरिया प्रजातींचे प्रमाणीकरण करण्यासाठी पॉलिमरेझ चेन रिॲक्शन पद्धतीचा वापर केला गेला.

तपासणी केलेल्या 30 फार्मपैकी 21 (70%) कॉक्सीडिओसिस संसर्गासाठी पॉझिटिव्ह आढळून आले त्यापैकी , 3 (14.28%) फार्ममध्ये केवळ आयमेरिया टेनेला प्रजाती आढळून आली, 11 (52.28%) फार्ममध्ये आयमेरिया एसरवुलिना ,आयमेरिया माइटिस आणि आयमेरिया टेनेला यांचा मिश्र संसर्ग दिसून आला आणि 7 (33%) फार्ममध्ये आयमेरिया एसरवुलिना आणि आयमेरिया माइटिसचा मिश्र संसर्ग दिसून आला.

पीसीआरद्वारे आण्विक ओळख आणि मॉर्फोमेट्रिवर आधारित सूक्ष्म ओळखीनंतर आयमेरियाच्या केवळ तीन प्रजाती प्रामुख्याने आयमेरिया एसरव्हुलिना आयमेरिया माइटिस आणि आयमेरिया टेनेला नागपूर विभागात आढळून आल्या.

या आजारावरील प्रचलित औषधी मदुरामायसिन च्या तुलनेत थायमॉल पावडरचे कोक्सीडिया विरोधी प्रभाव तपासले गेले. पॉलीथर आयनोफोरिक अँटीकोक्सीडियल मदुरामायसिनसोबत तुलना करता थायमॉलने तुलनात्मक अँटीकोक्सीडियल क्रिया प्रदर्शित केली.सदर अभ्यासाच्या आणि संशोधनाच्या निकालांनुसार थायमॉल पक्ष्यांच्या खाद्यातील रासायनिक अँटीकोक्सीडियल औषधींसाठी एक उत्तम पर्याय आहे.