OCCURRENCE AND IDENTIFICATION OF *EIMERIA* SPECIES IN CHICKEN, TURKEY AND QUAIL

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THESIS

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DEPARTMENT OF VETERINARY PARASITOLOGY

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DECLARATION

I hereby declare that this thesis entitled **"Occurrence and identification of *Eimeria* species in chicken, turkey and quail"** is a bonafide record of research done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, fellowship or other similar title, of any other University or Society.

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CERTIFICATE

Certified that this thesis, entitled **"Occurrence and identification of *Eimeria* species in chicken, turkey and quail"** is a record of research work done independently by Pooja G. Mankani (18-MVM-47), under my guidance and supervision and it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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We, the undersigned members of the Advisory Committee of **Pooja G. Mankani (18-MVM-47)**, a candidate for the degree of Master of Veterinary Science in Veterinary Parasitology, agrees that this thesis entitled **"Occurrence and identification of *Eimeria* species in chicken, turkey and quail"** may be submitted by **Pooja G. Mankani (18-MVM-47)** in partial fulfillment of the requirement for the degree.

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POOJA G. MANKANI

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Introduction

1. INTRODUCTION

The role of poultry industry is important in the socio-economic development of our country. The economics of poultry industry may be seriously affected due to faulty management of farms and outbreaks of various diseases. Among the most pathogenic parasitic diseases of poultry known for many years, coccidiosis still continues to be one of the most common and recognised diseases of poultry worldwide.

Avian coccidiosis, an enteric disease of poultry caused by multiple species of the genus *Eimeria*, is a major menace for poultry industry causing production losses, high mortality and morbidity rates. In developing countries like India, where farming is at low-grade, this disease becomes a major problem and causes heavy economic losses. The disease is an important cause of morbidity and mortality in other gallinaceous birds such as quail and turkey. However, the exact losses due to coccidiosis in India are not known because of lack of statistical indices.

Coccidiosis is caused by apicomplexan parasites of the genus *Eimeria*. In chicken seven distinct species of the genus *Eimeria* with different degrees of pathogenicity are recognised *viz*., *Eimeria tenella*, *E. necatrix*, *E. maxima*, *E. acervulina*, *E. brunetti*, *E. mitis* and *E. praecox*. Species of *Eimeria* that have been identified in quail are *E. uzura*, *E. bateri* and *E. tsunodai* while in turkey *E. meleagridis, E. dispersa, E. gallopavonis, E. meleagrimitis* and *E. innocua* are reported. Some species of *Eimeria* are highly pathogenic causing severe haemorrhagic enteritis with high mortality in young birds, whereas other species are slightly or moderately pathogenic.

During an outbreak of coccidiosis, the identification of the infection is usually done by considering the clinical signs, post mortem lesions and detecting the oocysts in faeces. The differentiation between the *Eimeria* species is conventionally carried out by using morphological methods such as oocyst morphology, morphometry, sporulation time, site of the intestine parasitized and location of the parasite in the intestinal epithelium. However, these methods are

not only time consuming but also unreliable due to overlapping of characteristics among the different species. Mixed infections also cause a problem for the discrimination of species. Polymerase chain reaction (PCR) assays have been developed for differentiation of *Eimeria* spp. using primers that specifically target different regions of the *Eimeria* genome such as ribosomal internal transcribed spacer 1 (ITS-1), ITS-2, 5S rRNA, the small subunit rRNA and sequence characterised amplified region (SCAR) markers. The sequences of ITS have contributed genetic markers for identifying the *Eimeria* spp. and primers targeting ITS-1 region have been developed for the identification of *Eimeria* spp. in chicken from tissue and faecal samples using PCR.

The preliminary prevalence study on *Eimeria* spp. in chicken have been performed in Kerala using conventional techniques (Gigi George, 1997). However, the use of molecular techniques for identification of *Eimeria* spp. have not been attempted so far in the region. Information regarding the occurrence of *Eimeria* spp. in chicken, turkey and quail in different management conditions and histopathological changes in these birds associated with coccidial infection is scanty in this region. Taking into consideration the above points, the present study has been undertaken with the following objectives,

1. Study the occurrence of *Eimeria* spp. in chicken, turkey and quail in organised poultry farms and backyard poultry units by conventional methods
2. Molecular confirmation of *Eimeria* spp. in chicken by polymerase chain reaction (PCR)

Review of literature

2. REVIEW OF LITERATURE

2.1 MORPHOLOGICAL IDENTIFICATION OF *EIMERIA* SPECIES

2.1.1 Morphology and Morphometry

Williams *et al*. (1996) recovered different species of *Eimeria* from commercially reared chicken in France using combinations of five methods *viz.,* oocyst morphology, enzyme electrophoresis, growth in embryonating eggs, characteristics of intestinal lesions and determination of pre-patent time. The results indicated multispecific infections in 95 per cent of the farms and the percentages of each species were: *E. acervulina* (100 per cent), *E. mitis* (82 per cent), *E. tenella* (77 per cent), *E. maxima* (73 per cent), *E. praecox* (45 per cent) and *E. brunetti* (27 per cent).

Castanon *et al*. (2007) developed a computational real time diagnostic tool COCCIMORPH, involving digital image study of sporulated oocysts of seven distinct species of *Eimeria* for identification.

Haug *et al*. (2008) studied the epidemiology of coccidial infections in commercial broiler flocks and compared the identification of *Eimeria* species by morphometric and PCR techniques. By using a standard McMaster technique the levels of oocyst per gram (OPG) were determined and the length of oocysts were measured by micrometry. The oocysts were categorised into three groups: an AM group (small oocysts, ≤18.8 µm; tentatively *E. acervulina* or *E. mitis*), an NTP group (medium-sized oocysts, 18.9 to 23.8 µm; tentatively *E. necatrix, E. tenella* or *E. praecox*) or a BM group (large oocysts, ≥23.9 µm; tentatively *E. brunetti* or *E. maxima*). However, it was observed that morphometric tentative identification and PCR were in complete agreement in only 49 per cent of the cases.

Carvalho *et al.* (2011) identified multiple species of *Eimeria* in broiler flocks using traditional morphological techniques. One hundred oocysts from each faecal

sample were photographed using a microscope Olympus BX 51 coupled Olympus DP71 camera and subsequently measured with the assistance of software Image-Pro Express 6.0. In the morphological analysis it was observed that, farms presented 100 per cent positivity for *E. brunetti*, *E. tenella* and *E. praecox* while *E. acervulina* was less frequent (63.3 per cent).

Muazu *et al.* (2008) conducted a survey on prevalence and identification of *Eimeria* species causing coccidiosis in poultry within Vom, Plateau State, Nigeria by examination wet mount smears of the faecal droppings, concentration technique for counting of oocyst, post-mortem examination of the dead birds and intestinal smear examination. Out of 200 faecal droppings and 100 post-mortem cases, 90 faecal samples and 30 post-mortem cases were found to be positive for *Eimeria* species.

Hadipour *et al*. (2011) identified the prevalence of *Eimeria* spp. based on the site of infecton, nature of gross pathological lesions induced and oocyst morphology. Out of 200 native chicken examined, 128 (64 per cent) were found positive for coccidiosis and four pathogenic *Eimeria* spp. were identified which included *E. tenella*, *E acervulina*, *E. necatrix* and *E. maxima*.

The identifying morphological characteristics of the oocysts of *E*. *necatrix* were to oblong ovoid shape without micropyle and the sporocysts measured about 8.16 to 13.26 µm in length and 5.12 to 6.12 µm in width (Jadhav *et al*., 2011).

Aljumaili (2013) compared qualitative method (faecal floatation) with wet smear for routine diagnosis of coccidia infection in broiler chicken and reported that the result of faecal floatation was more efficient and promising than that of wet smear.

The species identification of chicken *Eimeria* species from different poultry managements in South India was carried out based on the curvature characterisation, size, symmetry and internal structural characterisation of oocysts using

COCCIMORPH, the online diagnostic tool, which revealed the presence of *E. acervulina, E. tenella*, *E. mitis*, *E. maxima and E. necatrix* (Thenmozhi *et al*., 2014).

Gadelhaq *et al.* (2015) reported seven species of *Eimeria* from Egytptian baldi chicken. Oocysts of *Eimeria* species were harvested from intestines of naturally infected chicken and morphometric characterisation of oocysts was done with COCCIMORPH tool. The authors observed that COCCIMORPH tool recorded the same findings of microscopical examination.

2.1.2 Histopathology and Lesion scoring

Idris *et al*. (1997) examined the effects of varying doses of *E*. *maxima* in chicken by comparing the gross and microscopic lesion scores, body weight gain, feed conversion ratio and faecal oocyst count in Beltsville, Maryland. The results indicated that both gross and microscopic lesions were significantly affected by days post infection and oocyst dosage, whereas the lesion scores regressed with significant linearity on body weight gain.

Zulpo *et al*. (2007) studied the pathogenicity and histopathological observations of *E. tenella*, *E. acervulina*, and *E. maxima* in experimentally infected commercial broiler chicks in Londrina, Brazil. The major histopathological lesions induced by these three species were characterised by severe villous atrophy, multifocal areas of severe inflammatory infiltrate, marked proliferation of epithelial cells of crypts, dilatation and necrosis of submucosal glands, foci of discrete haemorrhage associated with various intralesional forms of the parasite. *Eimeria tenella* induced lesions were severe, *E*. *acervulina* induced lesions were moderate while *E*. *maxima* induced lesions were discrete.

Jenkins *et al*. (2008) studied the effect of *E*. *praecox* infection on concurrent *E*. *maxima* infection in susceptible chicken in Georgetown, United States and observed an average intestinal lesion score of 1.3 due to *E*. *maxima* compared to

minor lesions in chicken infected with *E. praecox*. Lesions due to *E. maxima* were numerous petechiae and thickening of the intestinal wall while in *E. praecox* infected chicken mucoid exudates were present. They analysed the oocysts excreted by the chicken in dual infection, which showed least effect by oocysts of *E. praecox* than those of *E. maxima*.

Patra *et al*. (2010) evaluated the histopathological changes in broiler chicken infected with *E. tenella* in a poultry farm at Mizoram. Post-mortem examination revealed distended caeca with bloody faeces. Histopathological study showed loss of epithelial tissue, necrosis of mucosa and submucosa of caecum, lymphoid hyperplasia, presence of cluster of oocysts and schizonts and mononuclear cell infiltration in mucosal layer.

You (2014) assessed the pattern of infection by *E. tenella*, *E. acervulina* and *E. maxima* in experimental broiler chicken in Korea. Histopathological examination revealed the presence of developmental stages of the parasites in duodenum, jejunum and caecum of experimentally infected chicken with *E. acervulina*, *E. maxima* and *E. tenella* respectively. The major histopathological changes observed were numerous intracellular schizonts in the epithelial cells of caecum, severe submucosal haemorrhage due to *E. tenella*, schizonts of *E. maxima* within the cells of villi of the jejunum and hyperplasia of villi due to *E. acervulina*.

Histopathological examination of caeca infected with *E. tenella* revealed, destruction of villi and presence of merozoites in the glandular as well as mucosal regions (Khaier *et al*., 2015).

Examination of the intestines for macroscopic lesions revealed the mean lesion scores of < 2+ in different intestinal portions while higher score of > 2+ was observed mainly to caeca in free range chicken in Northeast Tunisia (Kaboudi *et al*., 2016).

Histopathological examination of intestine affected with *Eimeria* spp. revealed loss of epithelial tissue, loss of villi, villous atrophy, crypt destruction, necrosis of submucosa, haemorrhage, cluster of oocysts in caecal mucosa and hyperplasia of lymphoid cells (Haile, 2018)

Melkamu (2018) conducted a study at Ethiopia to evaluate the pathogenicity, gross and histopathological lesions of coccidiosis in broiler chicken. Gross pathological lesions showed ballooning of the intestine and petechial haemorrhages and the observed lesion score ranged from +1 to +4 in infected chicken. Histopathological examination revealed different stages of parasite, inflammation, villous atrophy, marked proliferation of epithelial cells, plenty of oocysts and schizonts in lamina propria, necrosis of submucosa and desquamation of epithelium.

The prevalence of *Eimeria* spp. and its histopathological changes was studied by Shahraki *et al*. (2018) in chicken from different parts of Sistan. The prevalence rate was 20.95 per cent and histopathological lesions showed loss of epithelial tissue, hemorrhage, loss of intestinal villi and necrosis of the submucosa of the intestine and caecum. Several merozoites, schizonts were also found in the epithelial cells.

In a study on the histopathological changes in the intestine and caecum due to *E. tenella*, Helal *et al*. (2019) observed marked decrease in length of villi in ileum, tissue damage in caecum, haemorrhage and presence of coccidian oocysts in tissues in experimentally infected broilers. Additionally, the cells of crypts were highly invaded by the developmental stages of *E. tenella*.

2.2 MOLECULAR IDENTIFICATION OF *EIMERIA* SPECIES

Schnitzler *et al*. (1998) described a PCR based assay for the detection and differentiation of four pathogenic species of *Eimeria* in chicken. It was concluded that the Internal Transcribed Spacer-1 (ITS-1) region of *Eimeria* species contained sufficient inter-specific sequence variation to enable the selection of primers that

could be applied in PCR analysis to detect and differentiate between species which might provide excellent markers for epidemiological studies.

Woods *et al*. (2000) established high resolution electrophoretic procedures for the identification of *Eimeria* species using genetic markers in ribosomal DNA. The ITS-1 and ITS-2 regions of ribosomal DNA were amplified by PCR from DNA samples in five *Eimeria* species (*E. acervulina, E. brunetti, E. maxima, E. necatrix, E. tenella*), then subjected to denaturing polyacrylamide gel electrophoresis (D-PAGE) or single-strand conformation polymorphism (SSCP) analysis. The authors reported that D-PAGE differentiated the ITS-1 and ITS-2 regions in different species while SSCP allowed the detection of population variation between some isolates of *E. acervulina*.

Zhao *et al*. (2001) described a simple method for extracting DNA of *Eimeria* oocysts by dissolving in sodium hypochlorite and lysis buffer containing high concentration of EDTA, N-lauroylsarcosine and proteinase-K at 65ºC for 45 minutes before using the standard phenol-chloroform extraction technique. The results of PCR indicated that this method generated better quality DNA than the glass-bead grinding method and was suitable for both large or small number of living or dead oocyst samples.

Fernandez *et al.* (2003) reported the development of multiplex PCR assay using sequence characterised amplified region (SCAR) species specific markers for simultaneous detection and discrimination of *Eimeria* species in domestic fowl.

Lew *et al*. (2003) confirmed the presence of seven *Eimeria* species in chicken in Australia by comparing the ITS-1 sequences. The authors sequenced the ITS-1 region of Australian isolates of *Eimeria* and analysed the phylogeny and concluded that the ITS-1 was a good target for the development of species-specific assays.

Haug *et al*. (2007) evaluated different methods of DNA extraction and PCR

assays for identification of *Eimeria* spp. It was concluded that rupture of oocysts by minipestle grinding, extraction of DNA with GeneReleaser, followed by optimised single species PCR assays targeting species-specific ITS-1 sequences was a sensitive protocol for identifying *Eimeria* spp. from field samples.

Lien *et al*. (2007) characterised three species of *Eimeria* (*E. tenella*, *E. maxima* and *E. acervulina*) from chicken in Taiwan using PCR with primers specific for the ITS-2 region of ribosomal DNA (rDNA). The authors observed high nucleotide sequence identity (96.8 to 100 per cent) within a species after cloning and sequencing of the PCR products. In addition, ITS-2 nucleotide sequences for *E. tenella* had higher homology (98.5 to 99.3 per cent) than *E. maxima* (81.6 to 96.5 per cent) when compared with appropriate sequences deposited in GenBank.

Gupta (2009) reviewed different methods of diagnosis of poultry coccidiosis including conventional methods based on faecal examination, morphometry of sporulated oocysts, post-mortem lesions, histopathological analysis and PCR and concluded that the use of molecular methods proved to be very efficient for detection and differentiation of *Eimeria* species.

The molecular prevalence of seven species of *Eimeria* in chicken in Tamil Nadu was analysed by nested PCR targeting the ITS-1 region which revealed *E. necatrix*, *E. tenella* and *E. maxima* to be the prevalent species in broilers, while in layers the prevalent species were *E. necatrix*, *E. brunetti* and *E. maxima* (Aarthi *et al*., 2010).

Bhaskaran *et al*. (2010) reported the presence of multiple infections of *Eimeria* spp. with highest incidence of *E. tenella* followed by *E. mitis*, *E. acervulina*, *E.brunetti* and *E. maxima* from commercial poultry farms in India using ITS-1 PCR.

Hamidinejat *et al*. (2010) used PCR assay as well as parasitological examinations for the identification of *Eimeria* spp. in chicken around Khuzestan,

South-west Iran. The prevalence of *E. tenella*, *E. necatrix*, *E. acervulina*, *E. mitis* and *E. maxima* was recorded by both molecular examination using species-specific PCR and parasitological techniques. The authors observed that identification using traditional methods was not always conclusive for specific diagnosis of *Eimeria* species in chicken and that PCR based diagnosis resolved this problem.

Carvalho *et al.* (2011) utilised a combination of morphological, pathological and molecular markers for identifying multiple circulating species of *Eimeria* in broiler flocks and observed that samples containing at least 20 oocysts of each *Eimeria* spp. were found to be necessary for PCR amplification. It was concluded that PCR was suitable as a routine diagnostic technique for monitoring the fluctuations in field *Eimeria* populations.

In a study of chicken coccidiosis in broiler farms of Turkey, Guven *et al*. (2013) examined 624 faecal samples and observed all the seven species of *Eimeria* by morphological examination. However the results of species-specific PCR assay confirmed the presence of *E. maxima*, *E. acervulina*, *E. tenella*, *E. praecox* and nested PCR results showed the presence of *E. mitis* and *E. brunetti*. *Eimeria necatrix* was not differentiated in either of the PCR assays.

Rao *et al*. (2013) employed multiplex PCR assay using SCAR primers to detect *Eimeria* species in about 11 mixed species faecal isolates from various regions of Tamil Nadu and Andhra Pradesh. The species of *Eimeria* observed were *E. tenella* and *E. mitis* and multiplex PCR using SCAR primers was found to be highly useful in mixed infections under natural condition.

The presence of multiple copies of genomic targets and use of nested PCR strategy made ITS-1 based nested PCR more sensitive than multiplex PCR based on sequence characterised amplified region (SCAR) markers in the detection of *Eimeria* spp. in poultry (Kumar *et al.,* 2014).

Gadelhaq *et al*. (2015) was successful in complete rupturing of oocysts wall by freezing for 50 cycles in liquid nitrogen without use of sodium hypochlorite. Prepared samples were subjected for DNA extraction using commercial DNA kits and analysed through conventional PCR by using SCAR markers.

Prakashbabu *et al*. (2017) studied the occurrence of *Eimeria* spp. across different production systems using PCR and reported considerable diversity in the distribution of *Eimeria* spp. according to poultry unit size, system and management.

2.3 OCCURRENCE IN ORGANISED AND BACKYARD POULTRY FARMS

2.3.1 Occurrence in organised poultry farms

Gigi George (1997) conducted a preliminary prevalence study on *Eimeria* spp. in broiler chicken in Kerala. On visceral examination the prevalence was found to be 21.13 per cent and on faecal examination 10.65 per cent. The prevalence of coccidiosis was found to be higher during the month of September to November. *Eimeria tenella* was found to be the most prevalent species with prevalence rate of 72 per cent followed by *E. necatrix* (36 per cent) and *E. maxima* (1.33 per cent).

Haug *et al*. (2008) assessed the etiology and epidemiology of *Eimeria* species infection in commercial broiler flocks in Sweden. Occurrence of five *Eimeria* species identified by PCR targeting the ITS-1 region of the genome were *E. acervulina* (100 per cent), *E. tenella* (77 per cent), *E. maxima* (25 per cent), *E. praecox* (10 per cent) and *E. necatrix* (2 per cent).

Gustavo *et al*. (2010) evaluated the epidemiology of coccidiosis in broiler chicken raised in the Araguaína region, State of Tocantins, Brazil. From the parasitological examination, it was shown that 63.1 per cent of the sheds were positive for *Eimeria* species and the species identified were *E. maxima*, *E. acervulina*, *E. mitis* and *E. tenella*.

On-farm prevalence rate and management of chicken coccidiosis was studied by Hirani *et al*. (2011) in Gujarat by examining litter material from 318 farms and faecal droppings from 1911 birds which recorded the prevalence of coccidia in 22.44 per cent of the farms and 17.27 per cent of faecal droppings. Prevalence was higher under deep litter system rearing of layers than in broilers and cage rearing system.

Alemayehu *et al*. (2012) identified the prevalence of poultry coccidiosis in small and large scale production systems in Addis Ababa, Ethiopia. A total of 384 faecal samples were collected, examined and the results revealed that 89 samples (23.1 per cent) were positive for coccidia oocysts.

Al-Gawad *et al*. (2012) observed a total of 711 Egyptian Baldy Breed chicken of different ages and sex for the presence of *Eimeria* species. Microscopical identification of oocysts revealed 21.24 per cent of the chicken to be infected with five species of *Eimeria* namely *E. necatrix* (58.27 per cent), *E. tenella* (25.82 per cent), *E. acervulina* (19.20 per cent), *E. mitis* (10.59 per cent) and *E. maxima* (4.66 per cent). The highest rate of infection was noticed in winter season (45.13 per cent) and in 64 to 84 day old chicken (62.37 per cent).

Amare *et al*. (2012) examined a total of 638 dead White leghorn chicken of 1 to 60 days of age on post-mortem by gross lesions, mucosal scraping examination, histopathological study and morphometric identification of *Eimeria* species at Kombolcha poultry farm, Ethiopia. A prevalence rate of 22.3 per cent was observed and five *Eimeria* species were identified such as *E. tenella* (37.86 per cent), *E. brunetti* (29.22 per cent), *E. necatrix* (12.35 per cent), *E. acervulina* (15.22 per cent) and *E. maxima* (5.35 per cent).

Bachaya *et al*. (2012) conducted a survey on the detection and predominance of different *Eimeria* species causing coccidiosis in different localities of Muzaffargarh, Pakistan. They collected 500 gut samples of layer chickens along with 250 faecal droppings for the detection of different *Eimeria* species. Four species of

Eimeria i.e., *E. maxima* (30.20 per cent), *E. tenella* (39.93 per cent), *E. mitis* (19.13 per cent) and *E. necatrix* (10.74 per cent) were isolated from infected gut samples. The results showed that young layer chickens (60.16 per cent) had greater infection rate when compared to the adults (37 per cent). It was also reported that highest predominance of coccidiosis was found during the month of September (73.33 per cent) and lowest during April (42.86 per cent).

Dinka and Tolossa (2012) conducted a study on coccidiosis from November 2009 to April 2010 in broiler chicken aged six to ten months at Debre Zeit Agricultural Research Center (DZARC) poultry farm, Ethiopia. By mucosal scraping examination, gross and histopathological changes and identification of *Eimeria* species, it was recorded that 71.7 per cent of dead birds harboured coccidia infection. Highest incidence was reported for *E. tenella* (45 per cent) followed by *E. necatrix* (34.3 per cent), *E. acervulina* (31. 3 per cent) and *E. brunetti* (27.6 per cent).

Jadhav *et al*. (2012) studied the occurrence of coccidiosis in broiler chicken in Aurangabad region of Maharashtra. *Eimeria tenella* was found to be the most common species in 43.59 per cent of the positive samples and 12.67 percent of the total samples examined. Other species were *E. necatrix*, *E. brunetti*, *E. acervulina*, *E. praecox*, *E. maxima* and *E. mitis*. Besides these seven species, three new species namely *E. nikamae*, *E. tarabaie* and *E. shivpuri* were also reported.

Jatau *et al*. (2012) reported the prevalence of coccidial infection and preponderance of *Eimeria* species in free range indigenous and intensively managed exotic chicken in Nigeria. The overall prevalence was 33.3 per cent which included highest record in layers (44.3 per cent) followed by broilers (37.1 per cent) and indigenous chicken (18.6 per cent). Higher percentage of birds (69.2 to 84.6 per cent) had inapparent infection and 3.9 per cent to 7.69 per cent birds had severe infection. All the seven species of *Eimeria* were identified with highest prevalence of *E.*

maxima (58.6 per cent) followed by *E. acervulina* (47.1 per cent), *E. mitis* (30.0 per cent), *E. brunetti* (28.6 per cent), *E. tenella* (22.9 per cent), *E. necatrix* (15.7 per cent) and *E. praecox* (8.6 per cent). Mixed infections were common in the surveyed region.

Fornace *et al*. (2013) examined the occurrence of *Eimeria* species in small-scale commercial poultry farms in Ghana, Tanzania and Zambia. Faecal samples were collected from 73 small-scale (less than 2,000 birds per batch) intensive broiler and layer farms. Oocysts were detected in 86 per cent (63/73) of farms, multiple species were identified in 63 per cent (40/63) of the farms and up to six species were detected on a single farm. *Eimeria necatrix* was the highly pathogenic species detected in 21 per cent (15/73) of the farms surveyed. Additionally, OTU-Z-like sequences were also identified.

Thenmozhi *et al*. (2014) analysed a preliminary genetic diversity study on different isolates of *E. tenella* using the small subunit of 18S rRNA from different poultry management systems of South India and reported the highest incidence of *Eimeria* infection in commercial broiler breeders (88.475 per cent) followed by commercial broilers (62.856 per cent). The incidence reported in commercial layer, backyard poultry and the broiler poultry managements were 44.69 per cent, 44.44 per cent and 42.81 per cent, respectively. Study indicated varying degrees of divergence of *E. tenella* isolates from different management systems in chicken farms.

2.3.2 Occurrence in backyard poultry units

Ashenafi *et al.* (2004) investigated coccidiosis in scavenging indigenous chicken of central Ethiopia by clinical signs, post-mortem and microscopic examination and found 25.8 per cent of the chicken infected with coccidiosis. The species responsible for coccidiosis were *E. tenella, E. necatrix, E. maxima* and *E. acervulina.* There was a significant difference in prevalence of coccidiosis in different altitudes with 42.2 per cent prevalence in high altitude region followed by 21.5 per cent in mid altitude and 13.1 per cent as the least in low altitude regions.

A study on prevalence of *Eimeria* species in native chicken reared under semi-scavenging system and in the yard in Kazerun, Iran was conducted by Hadipour *et al*. (2011). Post-mortem and parasitological examination of 200 native chicken showed, 128 (64 per cent) to be infected with coccidiosis. *Eimeria tenella* was the most prevalent species (24 per cent) followed by *E. acervulina* (18 per cent), *E. necatrix* (12 per cent) and *E. maxima* (10 per cent).

Sharma *et al.* (2013) recorded a higher prevalence rate for coccidiosis in backyard poultry (53.61 per cent) when compared to organised farms (25.55 per cent) in Jammu, which was attributed to poor managemental practices and non-use of anticoccidiostats.

Garbi *et al*. (2015) conducted a study to determine the prevalence of coccidiosis in free ranging and intensively managed chicken in Ethiopia. Out of 384 chicken examined, the overall prevalence was 19.5 per cent and highest prevalence of 38.7 per cent was due to *E. tenella* and *E. acervulina* followed by *E. necatrix* (13.33 per cent) and *E. maxima* (9.33 per cent). Higher infection rate was observed in backyard chicken (27.6 per cent) compared to chicken under intensive management system (11.45 per cent). The prevalence of infection in backyard chicken may be high due to poor management practices, indiscriminate scavenging behaviour of free ranging chicken and non-use of coccidiostats.

Kaboudi *et al.* (2016) studied the prevalence of coccidiosis in free range chicken in Sidi Thabet, Northeast Tunisia. Six hundred and thirty faecal samples were collected from 15 flocks and 200 samples were found positive for oocysts of *Eimeria* spp. The overall prevalence rate of coccidiosis was 31.8 per cent and the prevalence rate of different species were *E. tenella* (61.5 per cent), *E. maxima* (12 per cent) and *E. acervulina* (1.5 per cent). Mixed *Eimeria* species infection was also observed in the study.

Lawal *et al*. (2016) conducted a survey from June 2014 to July 2015 to

investigate the prevalence of coccidiosis among local and exotic breeds of chicken in Maiduguri, Northeastern Nigeria by processing a total of 600 faecal samples using parasitological techniques. The overall prevalence rate was found to be 31.8 per cent and exotic breeds had high prevalence rate of 42.4 per cent and none was found among the local breeds. Based upon the husbandry system, the highest prevalence of 46.5 per cent was observed in the intensive system when compared with 10.0 per cent in the semi-intensive system.

The prevalence of chicken coccidiosis was estimated by Libssie and Bedada (2018) in and around Gondar town, Ethiopia. Out of 408 samples examined 24.3 per cent were detected as positive. Prevalence of coccidiosis in intensive system was 30.6 per cent and in semi-intensive system 19.6 per cent. In addition to the above, the prevalence among the housing types were 25.2 per cent in floor and 21.5 per cent in backyard.

Molla *et al*. (2018) conducted a study to estimate the prevalence of coccidial infection and to assess the risk factors in backyard chicken in Northwest Ethiopia. A total of 224 samples were examined by direct and floatation techniques and overall prevalence of coccidial oocysts was noted as 21.4 per cent (48/224). After analysing the data for sex, breed and age, male and local breeds were found more likely to be positive for coccidial infection when compared to female and exotic breeds. However, adult birds were identified to be more at risk of coccidial infection when compared to young ones.

A study to determine the prevalence of coccidiosis in poultry in Punjab-Pakistan was carried out by Yousaf *et al*. (2018). Out of 420 faecal samples, 23.80 per cent were positive for coccidian oocysts. Higher prevalence was observed in intensively reared poultry (28.57 per cent) compared to chicken under extensive management system (21.42 per cent).

Ketema and Fasil (2019) determined the prevalence of poultry coccidiosis in

intensive poultry and backyard chicken in Ethiopia. A total of 451 samples were collected and examined and the results revealed an overall prevalence of 19.5 per cent. Occurrence of coccidiosis in intensive management system was 20.6 per cent and in backyard poultry 17.9 per cent and this variation in prevalence was not statistically significant.

2.4 OCCURRENCE OF *EIMERIA* SPECIES IN TURKEY

Hein (1967) studied the pathogenic effect of *E. adenoeides* and *E. meleagrimitis* in three weeks old turkey poults in Weybridge, England. Infection with *E. adenoeides* caused haemorrhagic enteritis in the distal part of the small intestine and proximal part of the caeca and the lesions due to *E. meleagrimitis* noticed were at the proximal part of the small intestine. The study showed that the pathogenicity due to *E. adenoeides* was severe than that of *E. meleagrimitis*.

Long and Millard (1977) assessed the activity of anticoccidial drugs by examining 396 faecal samples from four turkey farms over two to ten weeks for the presence of oocysts in Huntingdon, England. The number of oocysts were low at two weeks, high at fourth week and declined by eighth week in sulphaquinoxaline and 3-nitro-4-hydroxyphenylarsonic acid treated poults. Oocyst numbers were low at second and fourth week, reached peak at sixth week and then declined in monensin treated turkey.

Ruff *et al*. (1981) studied the changes in the intestinal mucosa of turkey during the infection of *E. meleagrimitis*, *E. dispersa* and *E. adenoeides* in Beltsville, U.S.A. Malabsorption of glucose and methionine was seen with scanning electron microscopy (SEM) in some regions of the intestine of turkey infected with all three species. There were no differences in the muscle layers between infected and uninfected poults and the intestinal tissue dry weight was less in infected poults when compared with uninfected poults.

Augustine (1988) conducted an experimental study in poults of Beltsville small white and Nicholas turkey. They inoculated mixed strains of *E. adenoeides* and *E. meleagrimitis* in one, seven and fourteen days old poults and observed weight gain and feed conversion. In immunised poults there was little weight reduction and average feed conversions were 1.52 to 1.69 compared with that in unimmunised poults (2.98 to 5.14).

Ruff *et al*. (1988) examined 119 pen-raised turkey from nine states of United States. Out of which, 66 per cent were found to be positive for coccidiosis. The frequency of *E. meleagrimitis* was found to be high (97 per cent) followed by *E. gallopavonis* (47 per cent), *E. meleagridis* (27 per cent), *E. dispersa* (17 per cent), *E. innocua- E. subrotunda* (13 per cent) and *E. adenoeides* (7 per cent).

Droual *et al*. (1994) studied coccidiosis and necrotic enteritis in turkey flocks by necropsy in California. Gross pathology revealed necrotic enteritis at distal jejunum, ileum and caeca. Intestinal scrapings and histopathological examination revealed stages of coccidia in crypts and villi, presence of coccidial oocysts in the lumen and necrosis of the villi.

The pathogenic species of *Eimeria* infecting turkey (*E. adenoeides*, *E. meleagrimitis*, *E. gallopavonis*, and *E. dispersa*) were diagnosed by molecular method in United Kingdom and a set of primers were developed for PCR as diagnostic tools for identification of the species (Cook *et al*., 2010).

Poplstein and Vrba (2011) described strain variation of *E. adenoeides* in small turkey farms in Czech Republic. Two strains were isolated and cross protection between these two strains was confirmed. Analysis of 18S and ITS-1 sequences from Immucox®-T and Coccivac®-T revealed that each vaccine consisted of different strains of *E. adenoeides*.

Vrba and Pakandl (2014) characterised five species of *Eimeria* in turkeys by

traditional and molecular methods involving sequencing of 18S rDNA, COI and ITS-1 regions and phylogenetic analysis in Czech Republic. The species identified were *E. meleagridis, E. dispersa, E. gallopavonis, E. meleagrimitis* and *E. innocua*.

The host specificity of *Eimeria* species in turkey and chicken in Czech Republic was studied by Vrba and Pakandl (2015). They investigated the host specificity by using cross-trasmission experiments by inoculating six species of turkey and chicken *Eimeria* and monitoring oocysts production. The findings showed that the adaptation of the species to foreign hosts was possible.

El-Sherry *et al*. (2018) redescribed the morphological, biological and pathological features of *Eimeria* spp. in turkey in Germany. Oocysts dimensions of *E. meleagridis* and *E. gallopavonis* were found overlapping and macroscopic lesions induced by *E. meleagridis* and *E. adenoeides* were found similar. They summarised the features of *Eimeria* spp. infecting small intestine of turkey.

2.5 OCCURRENCE OF *EIMERIA* SPECIES IN QUAIL

Fisher and Kelley (1977) described *E. colini* species from bobwhite quail, *Colinus virginianus*. The oocysts were broadly ellipsoid and measured 24.8µm by 20.9µm, with a micropyle while polar granule and oocyst residuum were absent.

The species of genus *Eimeria* in Japanese quails were identified based on morphology and morphometry of the oocysts by Teixeira and Lopes (2002) in the State of Rio de Janeiro, Brazil. Four distinct species such as *E. bateri*, *E. uzura*, *E. tsunodai* and *E. fluminensis* were identified.

Teixeira *et al.* (2004) reported three species of *Eimeria viz*., *E. tsunodai, E. uzura* and *E. bateri* in Japanese quails in Brazil using morphological criteria and histopathological findings.

The intestinal *Eimeria* species in Japanese quails from different parts of Nenevah governorate were reported by Mohammad (2012). Out of 87 Japanese quails examined, 43 (49.4 per cent) were positive for *Eimeria* species. Three species identified were *E. tsunodai* (44.8 per cent), *E. uzura* (34.5 per cent) and *E. bateri* (24.1 per cent). The rate of infection was found to be high in young quails and low in adults.

Umar *et al*. (2014) described the species, gross and histopathological lesions of *Eimeria* in Japanese quails in Zaria. A total of 400 faecal samples were processed and the *Eimeria* species identified was *E*. *bateri*. Intestinal smear and histopathology revealed developmental stage (schizont) of the parasite in the caecum, desquamation of the epithelial lining with areas of necrosis and merozoites in the jejunum.

An investigation was carried out to determine the coccidial infection in Japanese quails in farms of Namakkal and Coimbatore districts by Anbarasi *et al*. (2016). They examined 76 faecal samples from three farms and the result revealed that 12 samples were positive for mixed infections of *Eimeria* species. The species identified were *E*. *bateri*, *E*. *tsunodai* and *E*. *uzura*.

Ahmed *et al*. (2017) examined 205 faecal samples from both migrant and farm's quails by direct and concentration method in Zagazig, Egypt. The results showed that the prevelance of *Eimeria* spp. was high in migrant quails (68.82 per cent) and low in farm's quails (23.21 per cent). Five species of genus *Eimeria* were identified in naturally infected migrant quails, *viz*., *E*. *bateri*, *E*. *tsunodai*, *E*. *uzura*, *E*. *colini* and *E*. *bahli*, and two species (*E*. *bateri* and *E*. *tsunodai*) were identified in farm's quails.

Arafat and Abbas (2018) identified the species of *Eimeria* in Japanese quail in Mansoura, Egypt. The samples from a total of 107 Japanese quail farms were collected and examined. Of which, 34 (31.78 per cent) farms were positive for *Eimeria* oocysts. The shape indices of sporulated oocysts were determined and four

Eimeria species were morphologically identified, *viz*., *E*. *bateri*, *E*. *tsunodai*, *E*. *uzura* and *E*. *minima*.

Simiyoon *et al*. (2018) studied the presence of *Eimeria* species in Japanese quails in Namakkal. Scrapings from the intestine and caeca were examined for the presence of unsporulated oocysts and morphometry of oocysts showed the presence of *E*. *bateri*. Histopathology of caecum showed necrosis of villi and developmental stages of *Eimeria* in the epithelium.

Elmorsy *et al*. (2020) identified *Eimeria* species of Japanese quail and studied the clinical effect of coccidiosis on birds. Fifty faecal samples were collected from 10 farms in and around Bhubaneswar, Odisha. All collected samples were positive for *Eimeria* and three species were isolated; *E*. *uzura* (86 per cent), *E*. *bateri* (58 per cent) and *E*. *tsunodai* (42 per cent). Three patterns of infection was observed in the study, such as single infection with *E*. *bateri* (16 per cent), single infection with *E*. *uzura* (42 per cent) and mixed infection of three species (42 per cent).

Materials and methods

3. MATERIALS AND METHODS

3.1 SAMPLE COLLECTION AND PROCESSING

Faecal samples of 300 chicken, 150 quail and 50 turkey were collected from organised poultry farms and backyard poultry units in and around Thrissur district during the period from June 2019 to December 2020. Out of this, 167 chicken, 150 quail and 50 turkey faecal samples were from organised farms and 133 chicken faecal samples were from backyard poultry units. Approximately 100 g of faecal sample was collected from each farm by "W" method (Kumar *et al*., 2014) and put into properly labeled polythene bags for transportation to the laboratory. Age group, vaccination status, feeding and management of the birds were recorded during collection of samples. Twenty-six intestinal samples of coccidiosis suspected chicken and two samples from quails were also collected from post mortem cases from the department of Veterinary Pathology, College of Veterinary and Animal Sciences, Mannuthy.

3.1.1 Processing of samples for detection of *Eimeria* spp.

The faecal samples and intestinal contents were processed by sedimentation and floatation techniques. The intestinal viscera were examined for gross pathological changes and lesions such as thickening, ballooning, presence of haemorrhage and white spots on the serosal surface of the different segments. Pieces of intestine showing lesions were cut, fixed in 10 per cent neutral buffered formalin and processed for histopathology. For isolation of DNA, intestinal tissue with lesions were cut, washed in phosphate buffered saline and stored at -20ºC. Sporulated oocysts were cleaned by repeated sedimentation in water, ruptured by vortexing with 0.5 mm glass beads and subjected to DNA isolation by phenol chloroform method.

3.1.1.1 Sedimentation Technique

Faecal samples and intestinal contents were homogenised in water using

mortar and pestle. The samples were then sieved and poured into 15mL centrifuge tubes and centrifuged at 2000 to 2500 rpm for 3 min. The supernatant was checked microscopically for any oocysts that might have failed to be pelleted before discarding. Sediment was examined for the presence of oocysts and subjected to sporulation.

3.1.1.2 Floatation Technique

Faecal samples, intestinal contents and scrapings were mixed with saturated saline using mortar and pestle. The samples were then sieved and poured into 15mL centrifuge tubes and centrifuged at 2000 to 2500 rpm for 3 min. After centrifugation 1mL of fluid from the top was transferred into another tube. Saturated saline was added to about 3/4th of the tube and again centrifuged. The step was repeated twice and 1 mL solution from the upper surface was taken and fresh tap water was added and centrifuged at 2500 rpm for 2 min. The pellet containing oocysts was washed thrice with tap water. Finally, the oocysts were washed with distilled water and the sediment obtained was subjected to sporulation.

3.2 MORPHOLOGICAL IDENTIFICATION OF EIMERIA SPP.

Morphological identification of *Eimeria* spp. in chicken and quail was done based on the shape of the oocysts, presence or absence of the micropyle and morphometry. The sporulation time was also recorded.

3.2.1 Sporulation of Oocysts

The washed oocysts were placed separately in Petri dishes and 2.5 per cent potassium dichromate solution was added. The Petri dishes were partially covered to allow the passage of oxygen and incubated at 37ºC for 24 to 72 h. The sporulation of the oocysts was confirmed by examination of a well mixed drop of suspension under the microscope. The sporulation time was recorded in each case.

3.2.2 Cleaning of Oocysts of *Eimeria* spp. after Sporulation

Oocysts culture were transferred into 15 mL centrifuge tube and centrifuged at 2000 rpm for 10 min. The supernatant was poured off into beaker and checked microscopically prior to discarding. Fresh tap water was added to the oocysts pellet, mixed and sedimented. The step was repeated twice or until all traces of the potassium dichromate were removed. After the final wash the cleaned oocysts was resuspended in a suitable volume of phosphate buffered saline and stored at 4ºC.

3.2.3 Morphological Examination

Morphological features such as shape of the oocysts and presence or absence of micropyle were recorded as per Soulsby (1982).

3.2.4 Micrometry

Micrometry was performed by measuring the length (L) and width (W) of the sporulated oocysts using a calibrated ocular micrometer mounted on a light microscope. A minimum of 10 oocysts was measured in each case and the average measurement was taken. The shape index (S.I.=L/W) for each oocyst type was also calculated and *Eimeria* species determination was done by considering the size and shape index.

3.3 OCCURRENCE OF *EIMERIA* SPP.

The occurrence rate of *Eimeria* spp. in chicken and quail in the study area was recorded. The influence of different areas, systems of management, age of the birds and seasonal effect on the occurrence of coccidiosis was analysed using Chi square test.

3.4 LESION SCORING

The intestine was thoroughly examined for gross pathological lesions and the observed lesions were assigned lesion scores from 0 to +4 according to the

system of Johnson and Reid (1970) (Table 3.1).

Table 3.1. Lesion scoring for intestinal and caecal lesions

Lesion score	Intestine	Caecum
0	No gross lesions	No gross lesions
1+	Small scattered petechiae and white spots on the serosa; no thickening of the intestinal wall; normal intestinal contents present.	Very few scattered petechiae on the caecal wall; no thickening of the caecal wall; normal caecal contents present.
2+	Numerous petechiae on the serosal surface; slight ballooning confined to the midgut area may be present; intestinal wall is somewhat thickened.	Lesions more numerous with noticeable blood in the cecal contents; cecal wall is somewhat thickened; normal cecal contents present.
3+	Extensive haemorrhage into the lumen of the intestine; serosal surface is covered with red petechiae. The serosal surface is rough and thickened with many pinpoint haemorrhages. Normal intestinal contents are lacking; ballooning extends over lower half of small intestine.	Large amounts of blood or caecal cores present; caecal walls greatly thickened.
4+	Extensive haemorrhage giving the intestine a dark colour; intestinal contents consist of red or brown mucus. Ballooning may extend throughout much of the length of the intestine.	Caecal wall greatly distended with blood or large caseous cores; faecal debris lacking or included in cores.

3.5 HISTOPATHOLOGICAL EXAMINATION

For microscopic evaluation of pathological changes, areas of intestine showing gross lesions were taken. Tissue samples of intestines, about 3 to 4 cm length were sampled and fixed in 10 per cent neutral buffered formalin and processed. The tissue samples were dehydrated in ascending grades of isopropyl alcohol, cleared in xylene, embedded in paraffin wax, sectioned at 4 µm thickness and stained with hematoxylin-eosin according to procedures described by Bancroft and Suvarna (2019). The stained sections were examined under light microscope for histopathological changes using 10X and 40X magnification.

The smears made with scrapings from the caecum were stained with Field stain (Pirehma *et al*., 1999) and examined microscopically for the developmental stages.

3.6 MOLECULAR IDENTIFICATION OF EIMERIA SPP.

3.6.1 Isolation of genomic DNA from the positive samples

Isolation of DNA was done from sporulated oocysts by phenol chloroform method (Sambrook and Russell, 2001) with few modifications. The DNA extraction from the tissue stages of coccidia was carried out using DNeasy Blood & Tissue Kit (QIAGEN, Germany).

3.6.1.1 Phenol Chloroform Method

3.6.1.1.1 Reagents

1. Phosphate Buffered Saline (PBS, 1 X solution), pH 7.2

Sodium chloride	8g
Potassium chloride	0.2g
Disodium hydrogen phosphate (Na_2HPO4)	1.133g
Potassium dihydrogen phosphate (KH_2PO4)	0.2g
Distilled water	1000 mL

   pH was adjusted to 7.2 by 1N NaOH, autoclaved at 121ºC for 15 min at 15 lbs pressure and stored at 4ºC until use.
2. Sterile No.8 glass beads (0.5 mm) (Sigma Aldrich, USA)
3. Phenol (Saturated, pH 7.8) (SRL, Mumbai)
4. Proteinase K (20 mg/mL in water) (QIAGEN, Germany)
5. Sodium Dodecyl Sulphate (SDS), 20%
   Dissolved 20 g of SDS in distilled water, made up to 100 mL, filtered and stored at room temperature.
6. Sodium chloride (5M)
   Dissolved 29.22 g of sodium chloride in 80 mL distilled water and made up the volume to 100 mL. Filtered the solution and stored at

room temperature.

7. Phenol: chloroform: isoamyl alcohol (25:24:1) (SRL, Maharashtra)
8. Chloroform
9. Ethanol 100%
10. Ethanol 70%
11. Double distilled water(DDW)
12. Tris EDTA (TE) buffer (pH 8.0)

    Tris base (10 mM) 1.2114g

    EDTA (0.1 mM) 0.3722 g

    Dissolved in 900 mL of distilled water and adjusted the pH to 8.0. Made up the volume to 1000 mL, filtered, autoclaved and stored at 4ºC.
13. Sodium acetate (3M)

    Dissolved 40.824 g of sodium acetate in 70 mL of distilled water and adjusted the pH to 5.5 with glacial acetic acid. Made up the volume up to 100 mL, autoclaved and stored at room temperature.

3.6.1.1.2 Protocol

Cleaned oocysts pellet were collected in a 15 mL centrifuge tube, centrifuged at 2500 rpm for 10 min and resuspended in 5 mL TE buffer for washing. It was centrifuged at 2500 rpm for 10 min, supernatant was removed and the pellet was resuspended in 2 mL TE buffer and kept on ice. Equal volume of sterile No.8 glass beads were added upto 2mL in a 15 mL centrifuge tube and the oocysts were smashed by high speed vortexing in several pulses, with intermittent chilling on ice followed by centrifugation at 1000 rpm for 5 min. The supernatant was collected and transferred to 15 mL falcon tube and stored on ice. The smashed oocysts were again flushed with 2 mL TE buffer by vortexing followed by centrifugation at 1000 rpm for 5 min. The supernatant was transferred to the previously collected tube, 0.33 volume of 10 per cent SDS and 5 µL proteinase K (20 mg/mL) was added and kept at 4ºC overnight at this point. Then it was centrifuged for 10 min at 2500 rpm and supernatant was recovered and placed in a

15 mL centrifuge tube. Subsequently equal volume of Tris saturated phenol was added, mixed by vortexing and centrifuged at 2500 rpm for 10 min. The aqueous phase was collected in a fresh tube and the phenol extraction step was repeated (in instances where the total volume fell below 4 mL, the volume was made upto 4 mL using TE buffer). Upper aqueous phase was transferred into a fresh tube and equal volume of phenol: chloroform: isoamyl alcohol was added, mixed by vortexing followed by centrifugation at 2500 rpm for 10 min. Equal volume of chloroform solution was added to the recovered aqueous phase, mixed by vortexing and centrifuged at 2500 rpm for 10 min. Aqueous phase was collected and the chloroform extraction step was repeated, to which 0.1 volume of 3M sodium acetate and two volumes of 100 per cent ethanol was added, vortexed and incubated at 70ºC for 1h. After incubation, the tube was centrifuged for 15 min at 2500 rpm and supernatant was removed. One volume of 70 per cent ethanol was added to the pellet was dissolved by inverting five times and centrifuged at 2500 for 10 min. Ethanol was removed and the pellet was allowed to air dry for 30 min. Then 50 µL of DDW was added to cover the pellet and allowed to resuspend overnight at 4ºC and the isolated DNA was stored at -20ºC.

3.6.1.2 Commercial kit Method

3.6.1.2.1 Reagents

1. DNeasy Blood & Tissue Kit (QIAGEN, Germany) contained the following solutions: Buffer ATL, Buffer AL, Buffer AW1 (Concentrate), Buffer AW2 (Concentrate), Buffer AE and Proteinase K
2. Absolute ethanol
3. Phosphate Buffered Saline (PBS), pH-7.2

3.6.1.2.2 Protocol

DNA was extracted from the intestinal tissue with the DNeasy Blood & Tissue Kit according to the manufacturer's protocol as follows:

1. The intestinal tissue was cut into small pieces and ground with small

amount of PBS using mortar and pestle. From this, 200 µL was taken and transferred to a 1.5 mL micro centrifuge tube. One hundred and eighty microliter of buffer ATL and 20 µL of proteinase K was pipetted into the micro centrifuge tube, mixed by vortexing and incubated overnight at 56ºC. The sample was vortexed for 15 sec directly before proceeding to step2.

2. Added 200 µL Buffer AL (without ethanol) to the sample, mixed thoroughly by pulse vortexing for 10 sec and incubated at 56ºC for 10 min.

3. Added 200 µL absolute ethanol to the sample and mixed thoroughly by pulse vortexing for 15sec.

4. Pipetted the mixture from step three into a DNeasy Mini spin column placed on a 2 mL collection tube provided with the kit and centrifuged at 8,000 rpm for one min. Discarded flow through and the collection tube.

5. Placed the DNeasy Mini spin column in a new 2 mL collection tube provided with the kit and added 500 µL Buffer AW1 and centrifuged for one min at 8,000 rpm. Discarded flow through and the collection tube.

6. Placed the DNeasy Mini spin column on a new 2 mL collection tube and added 500 µL Buffer AW2 and centrifuged for six min at 7,000 rpm to dry the DNeasy membrane. Discarded flow through and the collection tube.

7. Placed the DNeasy Mini spin column in a clean 1.5 mL micro centrifuge tube and pipetted 200 µL Buffer AE directly onto the DNeasy membrane. The solution was incubated at room temperature for one min and then centrifuged for one min at 8,000 rpm for elution.

8. Stored the extracted DNA at -20ºC until use.

3.6.1.3. Assessment of Concentration and Purity of DNA

The concentration and purity of isolated DNA were determined by measuring the absorbance of the samples at 260 and 280 nm using Nano Drop spectrophotometer (Thermo Scientific, USA).

3.6.2 Polymerase Chain Reaction

The DNA from *Eimeria* oocysts or intestinal tissue was amplified using *Eimeria* genus specific primers and species specific primers for *E. tenella*, *E. maxima*, *E. necatrix*, *E. brunetti*, *E. mitis*, *E. praecox* and *E. acervulina* in separate PCR reactions. The protocols for each reaction was standardised separately in a gradient thermal cycler (Bio-Rad T100, USA).

3.6.2.1 Reagents

1. PCR reaction buffer without $MgCl_2$ (10X) (Sigma Aldrich, Banglore)
2. *Taq* DNA Polymerase, 5U/µl (Sigma Aldrich, Banglore)
3. Deoxyribonucleotide triphosphate (dNTP) mix (10 mM each) (Thermoscientific, USA)
4. Magnesium chloride ($MgCl_2$) (25 mM) (Sigma Aldrich, Banglore)
5. Template DNA: (DNA extracted by phenol-chloroform method or DNA extraction kit)
6. Nuclease free water
7. Primers: Primers targeting ITS-1 region of *Eimeria* spp. was selected from Bhaskaran *et al*. (2010) (Table 3.2). The primers were custom synthesised from Sigma Aldrich, Bangalore. They were diluted with sterile nuclease free water to get a final concentration of 10 pmol/µL and stored at -20ºC.

Table 3.2. Primer sequences

Primer	Primer sequence	Product size(bp)
Eimeria spp. (universal primer)	F 5′AAGTTGCGTAAATAGAGCCCTC 3′ R 5′AGACATCCATTGCTGAAAG 3′	400-750
E. tenella	F 5′AATTTAGTCCATCGCAACCCT 3′ R 5′CGAGCGCTCTGCATACGACA 3′	278
E. necatrix	F 5′TACATCCCAATCTTTGAATCG 3′ R 5′GGCATACTAGCTTCGAGCAAC 3′	383
E. acervulina	F 5′GGCTTGGATGATGTTTGCTG 3′ R 5′CGAACGCAATAACACACGCT 3′	321
E. maxima	F 5′GCGGTTTCATCATCCATCATCG 3′ R 5′CGTTGTGAGAAGACTGAAAGGG 3′	145
E. brunette	F 5′GATCAGTTTGAGCAAACCTTCG 3′ R 5′TGGTCTTCCGTACGTCGGAT 3′	311
E. mitis	F 5′GGGTTTATTTCCTGTCGTCGTCTC 3′ R 5′GCAAGAGAGAATCGGAATGCC 3′	328
E. praecox	F 5′CCAAGCGATTTCATCATCGGA 3′ R 5′AAAGCAAAGCGATTCAAG 3′	116

3.6.2.2 PCR Protocol

3.6.2.2.1 Protocol for amplification using *Eimeria* genus specific primers

The reaction mixture and gradient cycling conditions were as given in Tables 3.3 and 3.4, respectively.

Table 3.3. Composition of reaction mixture for amplification using *Eimeria* genus specific primers

Components	Quantity (µL)
10 X PCR buffer (without $MgCl_2$)	2.5
dNTP (10 mM each)	0.50 (200 µM each)
Primer forward	1 (10 pmol)
Primer reverse	1 (10 pmol)
Magnesium chloride (25 mM)	1.50 (1.5 mM)
Taq polymerase (5 IU/µl)	0.20 (1U)
DNA template	5
Nuclease free water	13.3
Total	25

Table 3.4. Gradient PCR protocol for amplification using *Eimeria* genus specific primers

Steps	Temperature	Time	Cycles
Initial denaturation	94 ºC	4 min	1
Denaturation	94 ºC	30 sec	34
Annealing	53-58 ºC	30 sec	
Extension	72 ºC	30 sec	
Final extension	72 ºC	5 min	1

3.6.2.2.2 Protocol for *E. tenella*

The reaction mixture used was as given in Table 3.3 except for the primers. The gradient cycling conditions were as given in Table 3.5.

Table 3.5. Gradient PCR protocol for the detection of *E. tenella*

Steps	Temperature	Time	Cycles
Initial denaturation	94 ºC	4 min	1
Denaturation	94 ºC	30 sec	34
Annealing	56-63 ºC	30 sec	
Extension	72 ºC	30 sec	
Final extension	72 ºC	5 min	1

3.6.2.2.3 Protocol for *E. necatrix*

The reaction mixture used was as given in Table 3.3 except for the primers. The gradient cycling conditions were as given in Table 3.6.

Table 3.6. Gradient PCR protocol for the detection of *E. necatrix*

Steps	**Temperature**	**Time**	**Cycles**
Initial denaturation	94 ºC	4 min	1
Denaturation	94 ºC	30 sec	34
Annealing	55-62 ºC	30 sec	
Extension	72 ºC	30 sec	
Final extension	72 C	5 min	1

3.6.2.2.4 Protocol for *E. maxima*

The reaction mixture used was as given in Table 3.3 except for the primers. The gradient cycling conditions were as given in Table 3.7.

Table 3.7. Gradient PCR protocol for the detection of *E. maxima*

Steps	**Temperature**	**time**	**Cycles**
Initial denaturation	94 ºC	4 min	1
Denaturation	94 ºC	30 sec	34
Annealing	62.7-72.7 °C	30 sec	
Extension	72 ºC	30 sec	
Final extension	72 ºC	5 min	1

3.6.2.2.5 Protocol for *E. acervulina*

The reaction mixture used was as given in Table 3.3 except for the primers. The gradient cycling conditions were as given in Table 3.8.

Table 3.8. Gradient PCR protocol for the detection of *E. acervulina*

Steps	Temperature	Time	Cycles
Initial denaturation	94 ºC	4 min	1
Denaturation	94 ºC	30 sec	34
Annealing	60.7-70.7 ºC	30 sec	
Extension	72 ºC	30 sec	
Final extension	72 ºC	5 min	1

3.6.2.2.6 Protocol for *E. brunetti*

The reaction mixture used was as given in Table 3.3 except for the primers. The gradient cycling conditions were as given in Table 3.9.

Table 3.9. Gradient PCR protocol for the detection of *E. brunetti*

Steps	Temperature	Time	Cycles
Initial denaturation	94 ºC	4 min	1
Denaturation	94 ºC	30 sec	34
Annealing	60.7-70.7 ºC	30 sec	
Extension	72 ºC	30 sec	
Final extension	72 ºC	5 min	1

3.6.2.2.7 Protocol for *E. mitis*

The reaction mixture used was as given in Table 3.3 except for the primers. The gradient cycling conditions were as given in Table 3.10.

Table 3.10. Gradient PCR protocol for the detection of *E. mitis*

Steps	Temperature	Time	Cycles
Initial denaturation	94 ºC	4 min	1
Denaturation	94 ºC	30 sec	34
Annealing	62.2-72.2 ºC	30 sec	
Extension	72 ºC	30 sec	
Final extension	72 ºC	5 min	1

3.6.2.2.7 Protocol for *E. praecox*

The reaction mixture used was as given in Table 3.3 except for the primers. The gradient cycling conditions were as given in Table 3.11.

Table 3.11. Gradient PCR protocol for the detection of *E. praecox*

Steps	Temperature	Time	Cycles
Initial denaturation	94 ºC	4 min	1
Denaturation	94 ºC	30 sec	34
Annealing	58.6-68.6 ºC	30 sec	
Extension	72 ºC	30 sec	
Final extension	72 ºC	5 min	1

3.6.2.3 PCR Protocol for identification of Eimeria in quail

3.6.2.2.1 Protocol for amplification using *Eimeria* genus specific primers

The universal *Eimeria* genus specific primers were used for amplifying the ITS-1 region of *Eimeria* spp. in quails. The reaction mixture was as given in table 3.3 and the DNA isolated from oocysts and tissue lesions were used as templates.

The gradient cycling conditions for amplification were as given in Table 3.12.

Table 3.12. Gradient PCR protocol for amplification using *Eimeria* genus specific primers

Steps	Temperature	Time	Cycles
Initial denaturation	94 ºC	4 min	1
Denaturation	94 ºC	30 sec	34
Annealing	53-58 ºC	30 sec	
Extension	72 ºC	30 sec	
Final extension	72 ºC	5 min	1

3.6.3 Agarose Gel Electrophoresis

3.6.3.1 Reagents

1. Tris Borate EDTA (TBE) buffer (10 X) pH 8.2

   Trisbase 108.0 g

   Boricacid 55.0 g

   0.5 M EDTA, pH (8.0) 40mL

   Triple distilled water was added to make up the volume to one litre. This was autoclaved at 121ºC and 15 lbs pressure for 15 min and stored at room temperature. The stock solution was adjusted to 1X prior to use.

2. Agarose gel (1.5 per cent)

   Agarose low EEO (SRL) 1.5g

   TBE buffer (1X) 100 mL

3. Ethidium bromide stock solution

   Ethidium bromide(SRL) 10 mg

   Triple distilled water 1mL

   Mixed and stored in amber coloured bottles at 4ºC.

4. 100 bp DNA ladder (Sigma Aldrich, Bangalore)
5. 50 bp DNA ladder (Sigma Aldrich, Bangalore)

3.6.3.2 Protocol

The amplified PCR products were subjected to agarose gel electrophoresis in a submarine electrophoretic apparatus (Hoefer, USA). Electrophoresis of individual PCR product was done in 1.5 per cent agarose gel. The agarose gel was prepared by dissolving agarose in TBE buffer (1X) followed by boiling. Subsequently, it was cooled to 50ºC and ethidium bromide was added to a final concentration of 0.5 µg/mL and the gel was casted in the tray after placing the comb to create wells for loading the DNA. The PCR product was loaded into the well carefully and one well was loaded with DNA ladder. Electrophoresis was carried out at a constant volt of 5 volts per centimeter of gel until the bromophenol blue dye migrated to more than two third the length of the gel. Then the gel tray was taken out of the apparatus, visualised and photographed in a Gel-documentation system (Bio-Rad, USA).

3.7 NUCLEOTIDE SEQUENCE ANALYSIS

The PCR amplicons specific for *E. tenella*, *E. necatrix* and *E. maxima* were purified and sequenced at Agri Genome Labs Private Limited, Cochin, using Sanger's dideoxy nucleotide chain termination method. Column purification and custom bidirectional sequencing was done. The sequences were aligned using EMBOSS (*www.bioinformatics.nl>cgi-bin>merger*) and blasted using NCBI BLAST tool (*www.blast.ncbi.nlm.nih.gov>blast*) to analyse their similarity with other published sequences available in online databases and submitted to the GenBank through Bankit. Sequencing and nucleotide sequence analysis was also done for the ITS-1 PCR amplicons of *Eimeria* spp. from quails and the sequences submitted to GenBank.

3.8 PHYLOGENETIC ANALYSIS

For studying the intra species genetic diversity of different *Eimeria* species, the published ITS-1 sequences of isolates of different species of *Eimeria* were downloaded from GenBank database. The obtained sequences were aligned

with downloaded sequences using Cluster W programme of MEGA X software. Maximum likelihood method was used to interpret evolutionary history with 1000 bootstrap replications.

3.9 STATISTICAL ANALYSIS

Statistical analysis was done as described by Snedecor and Cochran (1994) using SPSS software version 24.0. The data related to occurrence of *Eimeria* spp. and the influence of factors like age, season and management systems on the occurrence of coccidiosis was analysed by Chi square test.

Results

4. RESULTS

4.1 SAMPLE COLLECTION AND PROCESSING

A total of 300 chicken, 150 quail and 50 turkey faecal samples collected from areas in and around Thrissur district were examined during the period from June 2019 to December 2020. Twenty six intestinal samples of coccidiosis suspected chicken and two samples from quail were collected from post- mortem cases from the department of Veterinary Pathology, College of Veterinary and Animal Sciences, Mannuthy. The faecal samples and intestinal contents were processed by sedimentation and floatation techniques and examined microscopically for coccidial oocysts. Intestines were examined for the gross lesions and the tissue samples were processed for histopathology. Sporulated oocysts and intestinal tissues were used for DNA isolation for molecular studies.

4.2 MORPHOLOGICAL IDENTIFICATION OF *EIMERIA* SPP.

Morphological identification of different *Eimeria* spp. in chicken and quail was done by a combination of morphological features of the oocysts (shape, presence or absence of the micropyle) and morphometry. The sporulation time was also recorded.

4.2.1 Sporulation of oocysts

The average sporulation time of *Eimeria* oocysts of chicken and quail were recorded and summarised in Table 4.1 and Table 4.2., respectively.

4.2.2 Morphology and micrometry of oocysts

In the present study, three species of *Eimeria* (*E. tenella*, *E. necatrix* and *E. maxima*) in chicken (Plate 1) and two species of *Eimeria* (*E. bateri* and *E. tsunodai*) in quail (Plate 2) were identified based on morphological features. The micrometry of the oocysts was done by measuring the length and width of sporulated oocysts (Plate 3) and shape index was calculated. The main morphological features and the measurement of oocysts of different *Eimeria* spp. in chicken and quail are summarised in Table 4.1 and Table 4.2., respectively.

4.2.2.1 Eimeria spp. in chicken

4.2.2.1.1 *Eimeria tenella*

Oocysts were broadly ovoid in shape, without micropyle and oocyst residual body and polar granule were present (Plate 1 A). The mean size of oocysts was 21 X 18.25 µm, the shape index was 1.15 and the sporulation time was 48 to72 h.

4.2.2.1.2 *Eimeria necatrix*

The oocysts were ovoidal in shape and the micropyle and oocyst residuum were absent. Polar granule was present and average size of oocysts was 23.77 X 22.05 µm (Plate 1 B). The shape index was 1.07 and sporulation time was 48 to 72 h.

4.2.2.1.3 *Eimeria maxima*

The oocysts were comparatively larger and broadly ovoidal in shape without micropyle. The oocyst residuum was absent, but polar granule was present (Plate 1 C). Sporulation time was 48 to 72 h and the average dimension of oocysts was 28 X 22.5 µm. The shape index was 1.24.

4.2.2.2 Eimeria spp. in quails

4.2.2.2.1 *Eimeria bateri*

Oocysts were sub-spherical without micropyle. Polar granule was present and the average size of oocysts was 23 X 15.4 µm (Plate 2 A). The shape index was 1.49 and the sporulation time was 24 to 48 h.

4.2.2.2.2 *Eimeria tsunodai*

Oocysts were ovoid, smaller in size with average measurement of 20 X 14.5 µm. Micropyle was absent and polar granule was present (Plate 2 B). Shape index was 1.37 and the sporulation time was 24 to 48 h.

Table 4.1. Morphology, micrometry and average sporulation time of *Eimeria* spp. in chicken

Species	Shape of the oocyst	Micropyle	Mean size of oocyst (µm)	Shape index	Avg. sporulation time (hrs)
E. tenella	Ovoid	Absent	21 X 18.25	1.15	48-72
E. necatrix	Ovoid	Absent	23.77 X 22.05	1.07	48-72
E. maxima	Broadly ovoid	Absent	28 X 22.5	1.24	48-72

Table 4.2. Morphology, micrometry and average sporulation time of *Eimeria* spp. in quail

Species	Shape of the oocyst	Micropyle	Mean size of oocyst (µm)	Shape index	Avg. sporulation time (hrs)
E. bateri	Sub-spherical	Absent	23 X 15.4	1.49	24-48
E. tsunodai	Ovoid	Absent	20 X 14.5	1.37	24-48

4.3 OCCURRENCE OF *EIMERIA* SPP.

4.3.1 Occurrence in Chicken

In this study, the overall occurrence of *Eimeria* spp. in chicken from 12 different areas in and around Thrissur was 37.66 per cent (113/300). On statistical analysis using Chi square test, the occurrence of coccidiosis in different areas was found to be significantly different ($p<0.01$) (Table 4.3).

Table 4.3. Occurrence of *Eimeria* spp. in chicken in and around Thrissur

Area	No. of samples	No. positive	% positive	p value
Organised Farms				
Poomala	22	-	-	**0.0036**
Pattikkad	20	12	60	
University Poultry Farm, Mannuthy	32	-	-	
Viruppakka	40	22	55	
Pazhuvil	28	07	25	
Changaramkulam	25	11	44	
Total	**167**	**52**	**31.13**	
Backyard Poultry Units				
Kunnamkulam	30	18	60	
Arangali	14	-	-	
Thalikkulam	20	17	85	
Madakkathara	20	-	-	
Kechery	23	15	65.21	
Amballur	26	11	42.30	
Total	**133**	**61**	**45.86**	
Overall Total	**300**	**113**	**37.66**	

Eimeria tenella was found to be the most predominant species with occurrence rate of 46.01 per cent followed by *E. necatrix* (39.82 per cent) and *E. maxima* (14.15 per cent) (Fig. 4.1) (Table 4.4). On statistical analysis using Chi square test, the occurrence rate of *E. tenella* was found to be significantly higher compared to the other two species ($p<0.01$).

Table 4.4. Species wise occurrence of *Eimeria* in chicken

Species	No. of samples examined	No. of samples positive	Percentage positive	p-value
E. tenella	113	52	46.01	**< 0.0001***
E. necatrix		45	39.82	
E. maxima		16	14.15	

*Highly significant (p<0.01)

4.3.1.1 Occurrence in different management systems

Samples were collected from six organised farms and six backyard poultry units in and around Thrissur. In organised farms the birds were maintained in deep litter system or cage system, fed with commercial feed and routinely vaccinated against viral diseases. The birds in backyard poultry units were maintained in free range system and were not routinely vaccinated. The rate of occurrence of *Eimeria* infection was found to be more in backyard poultry (45.86 per cent) and comparatively lesser in organised farms (31.13 per cent) (Fig. 4.2) (Table 4.5). On statistical analysis there was significant association between the occurrence of coccidiosis and the type of management (p<0.01).

Table 4.5. Occurrence rate of *Eimeria* spp. in chicken in different management systems

Management system	No. of samples examined	No. of samples positive	Percentage positive	p-value
Organised farms	167	52	31.13	**0.0002***
Backyard	133	61	45.86	
Total	**300**	**113**	**37.66**	

*Highly significant (p<0.01)

4.3.1.2 Age-wise Occurrence

The occurrence of *Eimeria* infection was observed to be higher in chicken aged more than six weeks (44 per cent) followed by the chicken aged one to three weeks (37.36 per cent) and three to six weeks (36.5 per cent) (Fig. 4.3) (Table 4.6). On statistical analysis there was no significant difference between the occurrence rates in different age groups.

Table 4.6. Occurrence rate of *Eimeria* spp. in different age groups in chicken

Age (in weeks)	**No. of samples examined**	**No. of samples positive**	**Percentage positive**	**p-value**
1-3	182	68	37.36	0.2119*(ns)
4-6	93	34	36.5	
>6	25	11	44	
Total	**300**	**113**	**37.66**	

*Not significant (p>0.05)

4.3.1.3 Season-wise Occurrence

The rate of occurrence of coccidiosis was found to be highest during monsoon (43.13 per cent) which decreased to 41.42 per cent during post-monsoon and 18.96 per cent in pre-monsoon (Fig. 4.4) (Table 4.7). The data was statistically analysed and the overall effect of season on the occurrence of coccidiosis was found to be highly significantly (p<0.01).

Table 4.7. Season-wise occurrence of coccidiosis in chicken

Season	**Number examined**	**Number positive**	**Percentage positive**	**p-value**
Pre-monsoon	58	11	18.96	**0.004***
Monsoon	102	44	43.13	
Post-monsoon	140	58	41.42	

*Highly significant (p<0.01)

4.3.2 Occurrence in quails

Quail faecal samples were collected from five different farms in and around Thrissur. However, infection with *Eimeria* spp. was identified in only one farm during the study period. The overall occurrence of coccidiosis in quail was found to be 16.66 per cent (Table 4.8).

Table 4.8. Occurrence of *Eimeria* spp. in quail in and around Thrissur

Area	**No. of samples examined**	**No. of positive**	**% positive**
Poomala	20	0	0
Pattikkad	30	0	0
University Poultry Farm, Mannuthy	40	0	0
Irinjalakuda	30	0	0
Vellakarithadam	30	25	83.33
Total	**150**	**25**	**16.66**

4.3.3 Occurrence in Turkey

A total of 50 faecal samples collected from six organised turkey farms in and around Thrissur were examined during the study period. *Eimeria* oocysts could not be detected in any of these samples (Table 4.9).

Table 4.9. Occurrence of *Eimeria* spp. in turkey in and around Thrissur

Area	**No. of samples examined**	**No. of positive**	**% positive**
Poomala	10	0	0
University Poultry Farm, Mannuthy	10	0	0
OJ Poultry, Thrissur	15	0	0
Chanoth	10	0	0
Pattikkad	05	0	0
Total	**50**	**0**	**0**

4.4 LESION SCORING

A total of 26 intestinal samples from coccidiosis suspected chicken were studied, out of which 14 samples were found to be positive for caecal coccidiosis and 12 for intestinal coccidiosis. The intestine was thoroughly examined for gross pathological lesions, type and location of the lesions were noted and the observed lesions were assigned lesion scores from zero (no gross lesion) to 4+ (most severe lesions) according to the system of Johnson and Reid (1970) (Table 3.1). The gross pathological lesions included ballooning of intestines (jejunum) with petechial hemorrhages, mixing of the contents with blood and mucus and thickened intestinal wall (Plate 4 A and B). The scrapings from the intestine revealed oocysts of *E. necatrix* and *E. maxima*. The observed average lesion score for intestinal lesions was 3+.

The gross pathological lesions of caeca included petechial haemorrhages, distended caeca filled with blood and thickened caecal wall (Plate 4 C and D). The caecal scrapings revealed oocysts of *E. tenella* and the observed average lesion score for caecal lesions was 2+.

Examination for gross pathological lesions in intestinal samples of quails revealed slight ballooning of the intestine and caeca, congested mucosa, numerous petechiae on the serosal surface with noticeable amount of blood in the contents (Fig 4.5.). The lesion score for intestine and caeca was 2+.

4.5 HISTOPATHOLOGY

The selected regions of intestine and caeca with lesions were processed for histopathology. The intestinal and caecal samples revealed characteristic lesions of coccidiosis. The histopathological section of jejunum showed marked destruction of villi, desquamation of epithelial cells resulting in villous atrophy, presence of schizonts in the intestinal epithelium and various developmental stages of coccidia in the border cells of villi (Plate 5). The histopathological section of caecum revealed sloughing and destruction of villi and presence of schizonts and immature oocysts in the epithelial cells (Plate 6). The caecal smears

showed presence of schizonts and merozoites (Plate 7). The schizonts were oval in shape filled with merozoites and the merozoites were found to be elliptical.

The histopathological section of intestine and caeca of quail revealed desquamation of epithelium of intestine and caeca with presence of various developmental stages of oocysts and destruction of villi (Plate 8).

4.6 MOLECULAR IDENTIFICATION OF *EIMERIA* SPP.

4.6.1 Isolation of genomic DNA from the positive samples

The genomic DNA was isolated from the sporulated oocysts and tissue samples by using phenol chloroform method and commercial DNA extraction kit, respectively. The concentration and purity of isolated DNA were determined by measuring the absorbance of the samples at 260 nm and 280 nm using Nano Drop spectrophotometer (Table 4.10 and 4.11).

Table 4.10. Concentration and purity of the isolated DNA samples from oocysts by phenol chloroform method

Species	Concentration (ng/µL)	A260/A280
E. tenella	143.6	1.91
E. necatrix	130.1	1.90
E. maxima	89.2	1.87

Table 4.11. Concentration and purity of the isolated DNA samples from tissue by commercial kit method

Species	Concentration (ng/µL)	A260/A280
E. tenella	58.2	2.14
E. necatrix	60.9	1.91
E. maxima	55.3	1.86

4.6.2 Polymerase Chain Reaction

The PCR protocol targeting ITS-1 region was standardised by gradient PCR using the genomic DNA samples and the genus specific and species specific primers from Bhaskaran *et al.* (2010) for *E. tenella*, *E. necatrix* and *E. maxima*.

4.6.2.1 Polymerase Chain Reaction for Eimeria Genus

The amplification conditions were standardised by gradient PCR using primers targeting ITS-1 region of *Eimeria* genus. A product of approximately 750 bp was amplified at annealing temperature ranging from 53°C to 58°C (Fig. 4.6). An annealing temperature of 57.3°C was selected for subsequent PCR.

4.6.2.2 Polymerase Chain Reaction for Detection of E. tenella

The gradient PCR was done to standardise the protocol for *E. tenella* using primers targeting ITS-1 sequence of *E. tenella*. A 278 bp product was amplified at an optimum annealing temperature of 63°C (Fig. 4.7).

4.6.2.3 Polymerase Chain Reaction for Detection of E. necatrix

The PCR was standardised using species specific primers targeting ITS-1 region of *E. necatrix*. A product size of 381 bp was amplified at an optimum annealing temperature of 62°C (Fig. 4.8).

4.6.2.4 Polymerase Chain Reaction for Detection of E. maxima

Gradient PCR was done for standardising the protocol for *E. maxima* using species specific primers targeting ITS-1 region. The 145 bp product was amplified at an annealing temperature of 63.7°C (Fig. 4.9).

No cross amplification was found among *E. tenella*, *E. necatrix* and *E. maxima* and mixed infection was not detected in any of the samples tested.

The PCR done on the same DNA templates using the species specific primers for *E. acervulina*, *E. brunetti*, *E. mitis* and *E. praecox* yielded no detectable amplified products. Out of the 26 tissue samples from chicken subjected to PCR, 14 caecal samples yielded positive results for *E. tenella* and 12 intestinal samples for *E. necatrix* DNA.

Of the 63 samples positive by conventional methods, 60 samples were confirmed by PCR, of which, 29 (48.33 per cent) were found to be positive for *E. tenella*, 23 (38.33 per cent) for *E. necatrix* and eight (13.33 per cent) for *E. maxima* (Table 4.12).

Table 4.12 Molecular identification of *Eimeria* spp. in chicken

Species	Number of samples positive by PCR	Percent positive
E. tenella	29	48.33
E. necatrix	23	38.33
E. maxima	08	13.33
Total	60	

4.6.3 Polymerase Chain Reaction for Detection of *Eimeria* spp. in Quail

The PCR protocol was standardised using primers targeting ITS-1 region of *Eimeria* genus. A product of approximately 600 bp was amplified at annealing temperature of 57.3°C for both oocyst and tissue DNA (Fig. 4.10).

4.7 NUCLEOTIDE SEQUENCE ANALYSIS

Column purification and custom bidirectional sequencing was done for the PCR amplicons of *E. tenella*, *E. necatrix*, *E. maxima* and *Eimeria* species from quails. The sequences were aligned using EMBOSS (*www.bioinformatics.nl>cgi-bin>merger*) and blasted using NCBI BLAST tool (*www.blast.ncbi.nlm.nih.gov>blast*) to analyse similarity with other published sequences.

4.7.1 Partial ITS-1 Sequences of *E. tenella*

A 278 bp partial ITS-1 sequence of *E. tenella* (Table 4.13) showed 100 per cent similarity with the other published sequences of *E. tenella* isolates (Accession numbers- MN830381.1, JX853831.1, GQ153635.1, AY779513.1 *etc*.) (Fig. 4.11).

4.7.2 Partial ITS-1 Sequences of *E. necatrix*

A 381 bp partial ITS-1 sequence of *E. necatrix* (Table 4.13) showed 97 per cent similarity with the published sequences of *E. necatrix* isolates (Accession numbers- LN609938.1, JX853833.1, LN609851.1 and LN609935.1 *etc*.) (Fig. 4.12).

4.7.3 Partial ITS-1 Sequences of *E. maxima*

A 145 bp partial sequence of ITS-1 region of *E. maxima* (Table 4.13) revealed 99 per cent similarity with other published sequences of *E. maxima* (Accession numbers- LN609863.1, JX853828.1, GQ153625.1 and FJ230374.1 *etc*.) (Fig. 4.13).

4.7.4 Partial ITS-1 Sequences of *Eimeria* spp. in quail

A 611 bp partial ITS-1 sequence of *Eimeria* spp. from Quail (Table 4.13) showed 95 per cent similarity with the other published sequences of *Eimeria* spp. (MG797522.1, MH620350.1, HQ680473.1and FJ449688.1 *etc*.) (Fig. 4.14).

The partial ITS-1 sequence of *E. tenella*, *E. necatrix* and *E. maxima* and *Eimeria* spp. in quails were submitted to Gen Bank using BankIt and accession numbers were obtained (Table 4.14).

Table 4.13. Partial ITS-1 sequences of *E. tenella*, *E. necatrix* and *E. maxima* and *Eimeria* spp. in quail

Species	Sequence	Product size
E. tenella	AATTTAGTCCATCGCAACCCTTGAATCTGTTTTTCTCTGCA ACGGTTTTTCTACTTTTTAAAATGGATGGAATTTTTTGCTG CTGCAAGGATATATAGCAGTAGTATGTACGTGGGCGATC GGGGGGGTGGTGGCGCATGCACGGGCTCGCGTGGGGTCT GTCGGTGGCAGCCCCAGCGCGCCGGCGCCAGCCCCGTGA TCGTCGATCGCGCACGTACGTGGAGGGGATTATGAGAGG AGAAGACGCGCACGGGGCTGTGTCGTATGCAGAGCGCTC G	278 bp
E. necatrix	TACATCCCAATCTTTGAATCGTTTTTTTTCAGCAACGATTTT TTTTCTATTAAAAAAAAAAAAGGGATATTTGTCTTGCGAG GCATGTGCTAGTAGTATGGGCGTGAGCGATGGGGGGGAAG TGGTGGTGCATGCGTGGGCTTGCTTGCATGTGTGGGGCTTG TCGGGGGCAGCCCCAGCGTGCCAGCCCCAGCCCCATGACC AGCCCCATAGTCGTCGATCACGACGAGGCGCGAGAATTAT GATGAGACTTGATCGGGGCTGTTTTTTTTTTTGCAGGACGT TCGCAGCTCGAGGGAATTCCGTGTTGTGTAGCGGTCTGTGT GCTGCTGTGCGATCGGTCTCTCTCTCCGCGTGCTCGTTGCT CGAAGCTAGTATGCC	381 bp
E. maxima	GCGGTTTCATCATCCATCATCGATCGGAAAAGCTTTTCTG GAGCGTTCTGCATTGGTGGGACTGTGGTGATGGGGGCTG CTGGGGGGAACGCCAGAGAACTAGCTAACCCAATCATTG AATCCCTTCTCAGTTCTTCTCACAACG	145 bp
Eimeria spp. in quail	GCGTAAATAGAGCCCTCTAAAGGATGCAAAAGTCGTAACA CGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTCACACA TTGCTCTTTACAGCTGCTTGATCGCCAATCTTTGCTCTAAG GCTTATTCTCTAACGTTCTGCATGCATGGGTGGCTGGTGGT GGAAGCCTCTGTGGGGAAGCATACGAAAACCTGTTTGGAC TAACCCCTTGCCTCATTTTTTTTTTTCCCACAACATTAATTT GAATGGATAAGATATTCTGAATGCCCCCTTGTCAGGGGAC GGGGTGATGATGCCCGCAGAGGATGGGGGGGGGGAGGACC GCGCGCCCTGTGGGGGCTGTGGGGGCTTTTGTGTTGTGGG GTGGGGGGTTGCGTGTTGGGTGGGCCCTGTGGGGGGCTCC CTAGGATCACCACCCATCCCCCCCCCCTGCGAAAATGAGG TCGTTACCTGTTATTTTATATTTAAAAAAAGGTAATGATTT CTTTTCAACTTTTTAAAGAAATGAACTTTTAGATGTGGAAG CTAAACGTTACATGCTGCTTGGAACTTTGCTTCAGCAAGAA ACTTTTGCTCACTAAGGTGAATCGAATCACTTTTGTTGATG AGCA	611 bp

Table 4.14. GenBank accession numbers for the partial ITS-1 sequences of *E.* tenella, *E. necatrix* and *E. maxima* and *Eimeria* spp. in quail

Sl. No.	Sequence	Accession number
1	*Eimeria tenella* Kerala isolate internal transcribed spacer 1, partial sequence	MW343727
2	*Eimeria necatrix* Kerala isolate internal transcribed spacer 1, partial sequence	MW353998
3	*Eimeria maxima* Kerala isolate internal transcribed spacer 1, partial sequence	MW349654
4	*Eimeria* species ITS-1 from Quail Kerala isolate	MW465639

4.8 PHYLOGENETIC ANALYSIS

4.8.1 Phylogenetic analysis of *E. tenella*

The evolutionary history using maximum likelihood method based on General Time Reversible model was analysed with 1000 bootstrap value. The corresponding gene sequences of *T. gondii* was used as an outgroup for analysis (Fig. 4.15). The phylogenetic analysis revealed that Kerala isolate of *E. tenella* occurred in same clade with corresponding isolates of Mathura, Bareily, Tamil Nadu and Andhra Pradesh and was distinct from the isolate of China.

4.8.2 Phylogenetic analysis of *E. necatrix*

The evolutionary history using maximum likelihood method based on General Time Reversible model was analysed with 1000 bootstrap value. The corresponding gene sequences of *T. gondii* was used as an outgroup for analysis (Fig. 4.16). On phylogenetic analysis, the Kerala isolate of *E. necatrix* was found to occur as a distinct clade from corresponding isolates of China, UK, Australia and Faizabad.

4.8.3 Phylogenetic analysis of *E. maxima*

The evolutionary history using maximum likelihood method based on General Time Reversible model was analysed with 1000 bootstrap value. The corresponding gene sequences of *T. gondii* was used as an outgroup for analysis

(Fig. 4.17). The phylogenetic analysis revealed that Kerala isolate of *E. maxima* occurred in same clade with isolates of Australia, Tamil Nadu and China and was in sister clade to the isolate of Faizabad.

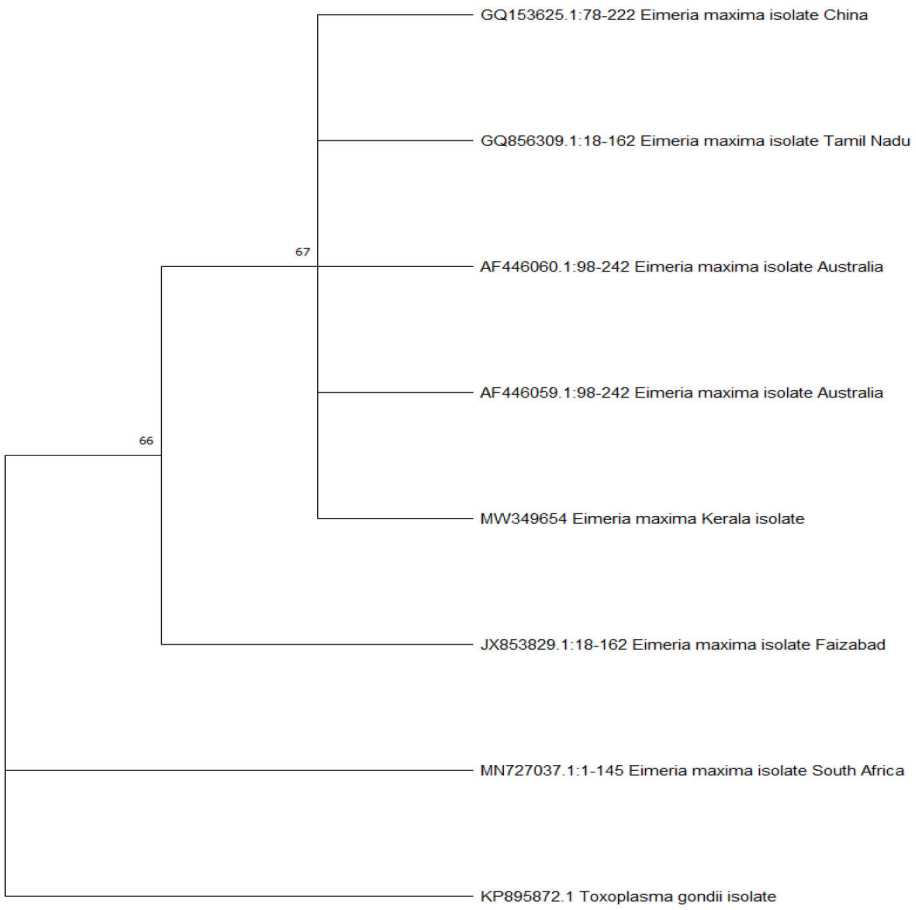

Fig. 4.17 Phylogenetic tree based on ITS-1 region of *E. maxima*

Phylogenetic analysis was done in MEGA X software, by using the Maximum Likelihood method based on the General Time Reversible model, and Gamma distributed with invariable sites (G + I). The codon positions 1st, 2nd, 3rd and non-coding were included. All positions containing gaps and missing data were eliminated

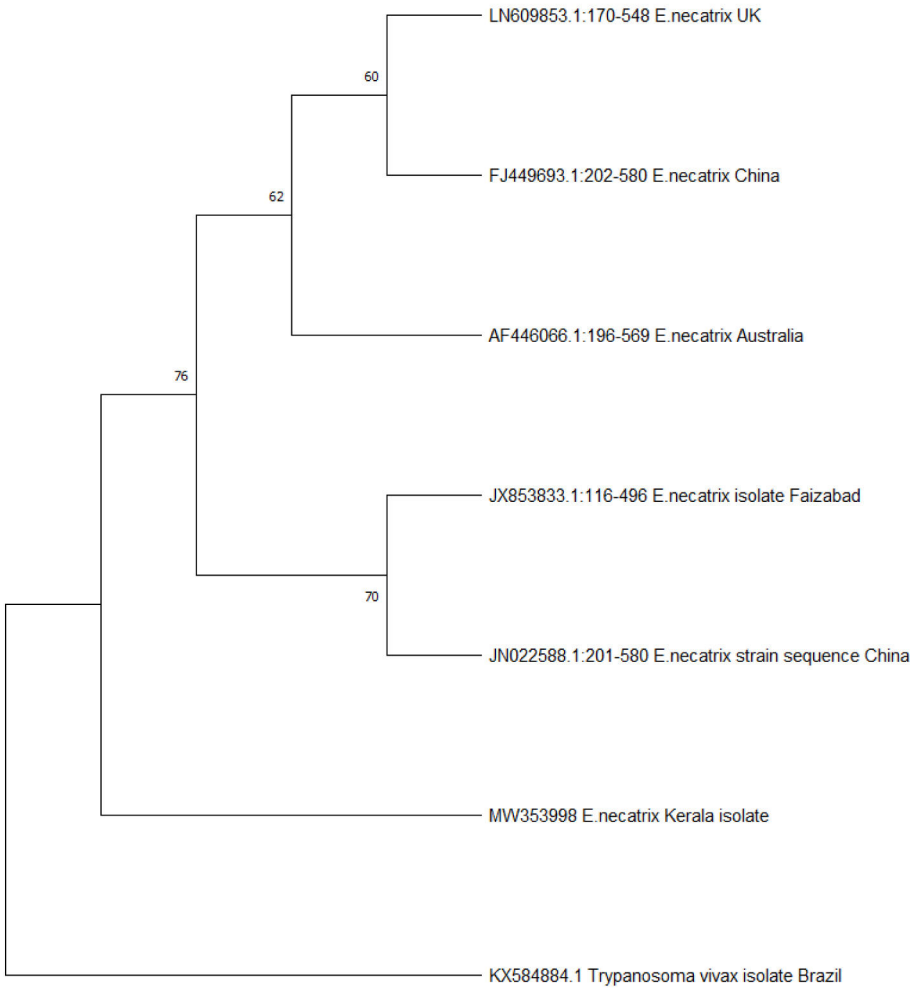

Fig. 4.16 Phylogenetic tree based on ITS-1 region of *E. necatrix*

Phylogenetic analysis was done in MEGA X software, by using the Maximum Likelihood method based on the General Time Reversible model, and Gamma distributed with invariable sites (G + I). The codon positions 1st, 2nd, 3rd and non-coding were included. All positions containing gaps and missing data were eliminated

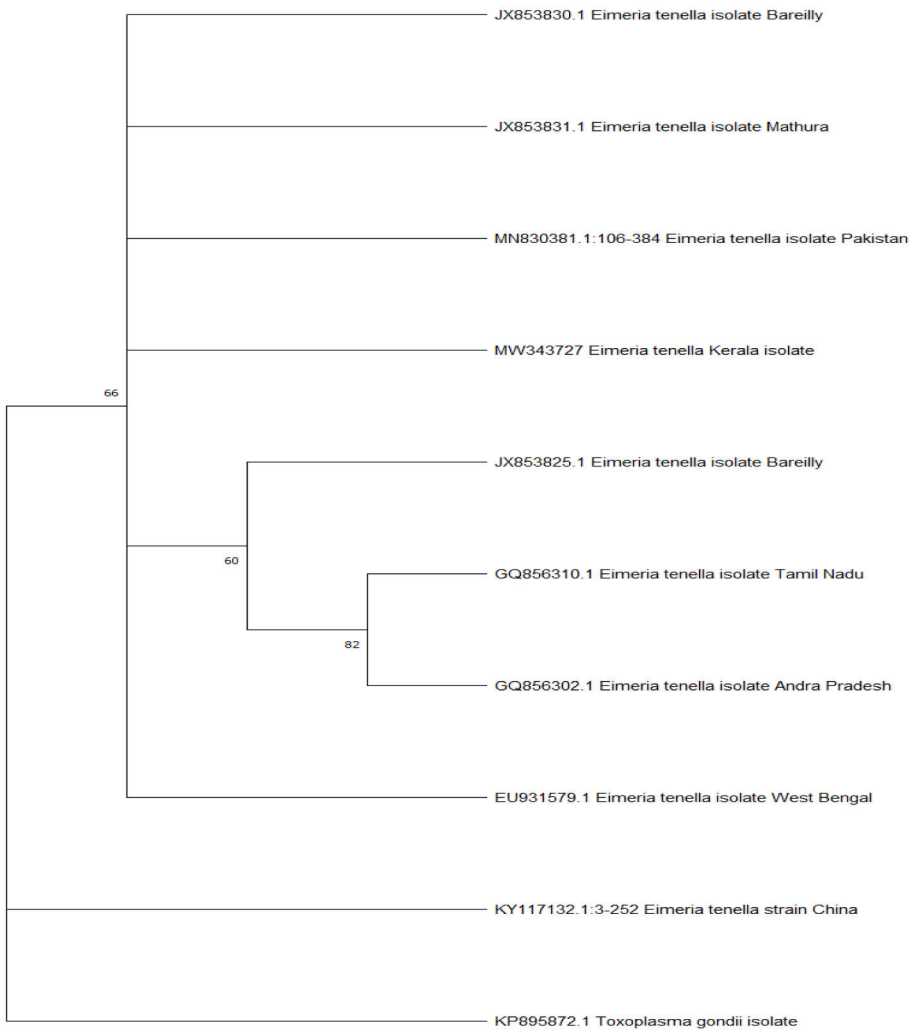

Fig. 4.15 Phylogenetic tree based on ITS-1 region of *E. tenella*

Phylogenetic analysis was done in MEGA X software, by using the Maximum Likelihood method based on the General Time Reversible model, and Gamma distributed with invariable sites (G + I). The codon positions 1st, 2nd, 3rd and non-coding were included. All positions containing gaps and missing data were eliminated

Discussion

5. DISCUSSSION

5.1 SAMPLE COLLECTION AND PROCESSING

The occurrence of coccidiosis in chicken in different management systems was studied by examining a total of 300 faecal samples from organised poultry farms and backyard poultry units from areas in and around Thrissur district. All the 150 samples from quail and 50 samples from turkey were from organised farms. Twenty six intestinal samples of coccidiosis suspected chicken and two samples from quail collected from post mortem cases were also examined during the study period. All the samples were examined microscopically for morphological identification of *Eimeria* spp. Sixty three samples from chicken that were positive by conventional methods were subjected to ITS-1 PCR for molecular confirmation of *Eimeria* species.

5.2 MORPHOLOGICAL IDENTIFICATION OF *EIMERIA* SPP.

During this study the morphological identification of *Eimeria* spp. in chicken and quail was done by studying the morphological features of oocysts, morphometry and sporulation time.

5.2.1 Sporulation of oocysts

The average sporulation time of *Eimeria* oocysts recovered from chicken and quail faecal samples was recorded. The sporulation time of *E. tenella*, *E. necatrix* and *E. maxima* oocysts was 48 to 72 h which was in accordance with Soulsby (1982). The sporulation time of *E. bateri* and *E. tsunodai* was 24 to 48 h which corroborated with the observation of Fisher and Kelley (1977) who also noted the sporulation time to be 48 h for these species.

5.2.2 Morphology and micrometry of oocysts

Three species of *Eimeria* in chicken and two species of *Eimeria* in quail were identified based on morphological features. The oocysts of *E. tenella* and

E. necatrix were ovoid in shape whereas that of *E. maxima* were broadly ovoid. These findings are in agreement with Soulsby (1982). On morphometry, *E. necatrix* oocysts measured 23.77 X 22.05 µm and had a shape index of 1.07, while *E. maxima* oocysts were 28 X 22.5 µm in size with shape index of 1.24 and *E. tenella* oocysts measured 21 X 18.25 µm with shape index of 1.15. These findings concurred with many of the previous studies. Khaier *et al*. (2015) reported the mean size of *E. tenella* oocyst to be 19.63 X 17.02 µm with shape index of 1.15 while Jadhav *et al*. (2011) observed the mean values of *E. necatrix* oocyst measurement to be 13.2 to 22.5 µm in length and 11.0 to 18.7 µm in width. Similarly, Al-Gawad *et al*. (2012) reported the mean size of *E. maxima* oocyst to be 29.9 X 23.8 µm with a shape index of 1.25. In a study conducted in Tamil Nadu, Rao *et al*. (2013) recorded mean morphometric values of 22 X 18 µm for *E. tenella* oocyst, 20.8 X 17.5 µm for *E. necatrix* oocyst and 31.19 X 18.5 µm for *E. maxima* oocyst. In this study it was observed that the morphological features of the oocysts as well as the morphometrical values overlapped for the three species, thus making the speciation based on these characters difficult.

The oocysts of *E. bateri* in quail were subspherical in shape and *E. tsunodai* were ovoid. The mean morphometric values of the oocysts of *E. bateri* was 23 X 15.4 µm with the shape index of 1.49 and that of *E. tsunodai* was 20 X 14.5 µm with shape index of 1.37. Similar observations were made by Mohammad (2012) who reported the mean size of *E. tsunodai* oocyst to be 19 to 14.6 µm and that of *E. bateri* oocyst to be 22 to 16.4 µm.

5.3 OCCURRENCE OF *EIMERIA* SPP.

5.3.1 Occurrence in chicken

The overall occurrence of *Eimeria* spp. in chicken in and around Thrissur was 37.66 per cent (113/300). *Eimeria tenella* was found to be the most predominant species with occurrence rate of 46.01 per cent which was significantly higher compared to that of *E. necatrix* (39.82 per cent) and *E. maxima* (14.15 per cent). These findings agree with many of the previous reports.

Eimeria tenella was found to be the most prevalent species of *Eimeria* in poultry in a previous study conducted by Gigi George (1997) in Kerala. Similarly, Bhaskaran *et al*. (2010) also reported the incidence of *E. tenella* to be higher when compared to *E. necatrix* and *E. praecox* in Tamil Nadu. In the studies conducted in different farms in Ethiopia, Amare *et al*. (2012) and Dinka and Tolossa (2012) reported higher prevalence for *E. tenella* compared to other *Eimeria* species in poultry.

5.3.1.1 Occurrence in different management systems

In the study, the rate of occurrence of *Eimeria* spp. infection was found to be significantly higher in backyard poultry (45.86 per cent) compared to that in organised farms (31.13 per cent). The findings were in accordance with Sharma *et al.* (2013) who recorded a higher prevalence rate for coccidiosis in backyard poultry (53.61 %) when compared to organised farms (25.55 %) in Jammu, which was attributed to poor managemental practices and non-use of coccidiostats. Similarly, Garbi *et al*. (2015) reported higher rate of infection in backyard chicken (27.6 %) compared to chicken under intensive management system (11.45 %) in Ethiopia and opined that the prevalence of infection in backyard chicken may be high due to poor management practices, indiscriminate scavenging behaviour of free ranging chicken and non-use of coccidiostats. The higher occurrence rate observed in backyard poultry in this study could be attributed to poor management practices and lesser use of anticoccidials. Moreover, the humid climatic conditions prevailing in the state favour rapid sporulation of oocysts and quick transmission of disease. However, the findings were in contrast with the observtion of Ketema and Fasil (2019) who reported higher occurrence rate of coccidiosis in intensive management system (20.6 per cent) compared to that in backyard poultry (17.9 per cent) in Ethiopia. This was attributed to the rearing of chicken in deep litter system, which provided optimal temperature and humidity for the sporulation of oocysts. Other factors like overcrowding and water leakage also might have contributed to higher occurrence rate.

5.3.1.2 Age-wise Occurrence

There was no significant difference between the occurrence of *Eimeria* spp. infection in birds belonging to different age groups. However, the occurrence rate was relatively higher in chicken aged above six weeks (44 per cent) compared to the chicken aged one to three weeks (37.36 per cent) and three to six weeks (36.5 per cent). Similarly, Al-Gawad *et al*. (2012) recorded the highest rate of infection in 64 to 84 day old Egyptian native breed chicken and attributed the difference in age susceptibility between the native breed and normal broilers to genetic factors. The higher occurrence rate observed in birds aged above six weeks in the present study could be due to the lesser number of samples examined in this age group.

5.3.1.3 Season-wise Occurrence

The rate of occurrence of coccidiosis was significantly higher during monsoon compared to the other seasons. Similarly, in a study in Pakistan, Bachaya *et al*. (2012) reported the highest occurrence of coccidiosis during the month of September (73.33 per cent) and lowest during April (42.86 per cent) and attributed it to the high level of humidity and temperature in these months of the year. The findings of the present study disagree with the reports of Al-Gawad *et al*. (2012) who reported the highest rate of infection in winter season (45.13 per cent) in Egypt. The higher occurrence rate in monsoon observed in the present study could be due to high rainfall with consequent increase in humidity which provides a favourable environment for rapid sporulation of oocysts.

5.3.2 Occurrence in Quail

The overall occurrence of *Eimeria* spp. in quail in and around Thrissur was 16.66 per cent (25/150) and two species of *Eimeria*, *E. bateri* and *E. tsunodai* were identified based on oocyst morphology and morphometry. In a study conducted in the farms of Namakkal and Coimbatore, Anbarasi *et al*. (2015) reported *E. bateri*, *E. tsunodai* and *E. uzura* in Japanese quails. Similarly,

Elmorsy *et al*. (2020) identified *E. bateri*, *E. tsunodai* and *E. uzura* in Japanese quails in Odisha. We could not encounter any previous report on *Eimeria* spp. in quails in Kerala and as such this forms the first report of *Eimeria* spp. in quails from Kerala.

5.3.3 Occurrence in Turkey

Oocysts of *Eimeria* spp. could not be detected in any of the 50 faecal samples collected from six organised turkey farms in and around Thrissur. The negative results obtained might be due to the lesser number of samples examined and the lesser turkey population in this area.

5.4 LESION SCORING

The gross pathological lesions observed in the present study included distended intestine and caeca, petechial hemorrhages, blood and mucus in the contents and thickened intestinal and caecal walls. The observed average lesion score was 3+ for intestinal lesions and 2+ for caecal lesions. Similar findings were reported by Al-Gawad *et al*. (2012) who observed the lesion score of +2 to +4 in caecal lesions and +2 to +3 in intestinal lesions. The gross pathological changes observed in the present study were in accordance with the findings of Melkamu (2018), who observed ballooning of the intestine, enlarged and distended caeca filled with blood and petechial haemorrhages with lesion scores ranging from +1 to +4.

In quail, the gross pathological lesions of intestine and caeca included slight ballooning, congestion in the mucosa, numerous petechiae on the serosal surface with noticeable amount of blood in the contents. These observations were in accordance with the reports of Elmorsy *et al*. (2020) who observed ballooning in the small intestine and caeca with thickened intestinal mucosa in quails.

5.5 HISTOPATHOLOGY

Histopathological examination of sections from the intestine and caeca

revealed desquamation of epithelial cells resulting in villous atrophy and destruction of the villi with various developmental stages of coccidia in the epithelium. The schizonts were oval in shape filled with merozoites and the merozoites were elliptical. The immature oocysts were also detected in the epithelial cells. The findings were in accordance with many of the previous reports. You (2014) observed the presence of developmental stages of the parasites in the intestine and caecum, severe submucosal haemorrhage due to *E. tenella* and schizonts of *E. maxima* within the cells of villi. Similarly, Khaier *et al*. (2015) observed the presence of merozoites in the glandular region and the mucosal region with destruction of villi. Shahraki *et al*. (2018) reported loss of epithelial tissue, haemorrhage, loss of intestinal villi and necrosis of the submucosa of the intestine and caecum with several merozoites and schizonts in the epithelial cells. Helal *et al*. (2019) noticed marked decrease in the length of villi in ileum and caecum, haemorrhage and presence oocysts in the tissue. The observed histopathological changes in the present study may be due to the most pathogenic stage, the second generation schizonts, which caused excessive tissue damage, bleeding and destruction of the mucosa and muscularis layer.

The histopathology of quail intestinal and caecal sections revealed desquamation of epithelium with various developmental stages of oocysts and destruction of villi. The mucosa, epithelial crypts and villi were damaged due to proliferation of the endogenous stages of coccidia. These findings were in accordance with Elmorsy *et al*. (2020) who observed desquamation of mucosal villi of small intestine with the presence of oocysts. Similarly, Simiyoon *et al*. (2018) observed desquamation of intestinal villi along with various developmental stages of schizonts in *Eimeria* infection in quails.

5.6 MOLECULAR IDENTIFICATION OF *EIMERIA* SPP.

5.6.1 Isolation of genomic DNA from the positive samples

In the present study the genomic DNA was isolated from the sporulated oocysts by rupturing the oocysts using sterile No.8 glass beads (0.5 mm) followed

by phenol chloroform method while commercial DNA extraction kit was used to extract DNA from tissues. Haug *et al*. (2007) described the rupture of oocysts by minipestle grinding using glass beads (0.5 mm) and extraction of DNA with GeneReleaser to be efficient. Zhao *et al*. (2001) described a different method for extracting DNA of *Eimeria* oocysts by dissolving in sodium hypochlorite and lysis buffer containing high concentration of EDTA, N-lauroylsarcosine and proteinase-K at 65ºC for 45 minutes before using the standard phenol chloroform extraction technique which yielded better quality DNA than the glass bead grinding method. Carvalho *et al*. (2011) observed that phenol chloroform method was more efficient compared the commercial kit method for extracting DNA from oocysts of *Eimeria* spp.

5.6.2 Polymerase Chain Reaction

In the current study PCR assay targeting ITS-1 region was used for genus specific and species specific amplification of *E. tenella*, *E. necatrix* and *E. maxima*. Many authors have explained ITS-1 sequence to be a good target for the species specific identification of *Eimeria* species. Schnitzler *et al*. (1998) observed that the ITS-1 region of *Eimeria* species contained sufficient inter specific sequence variation to detect and differentiate between species. Lew *et al*. (2003) reported that the ITS-1 was a good target for the development of species specific assays. Kumar *et al*. (2014) reported that ITS-1 based PCR assay to be more sensitive than multiplex PCR based on SCAR markers due to the presence of multiple copies of genomic targets in the cells.

5.6.2.1 Eimeria Genus

The primers targeting ITS-1 region of *Eimeria* genus, amplified a product of approximately 750 bp at an annealing temperature of 57.3°C. The results were in accordance with those of Haug *et al*. (2007) and Bhaskaran *et al*. (2010) who used the same set of primers in their studies for molecular identification of *Eimeria* spp.

5.6.2.2 Eimeria tenella

Primers for *E. tenella* targeting ITS-1 sequence amplified a 278 bp product at an optimum annealing temperature of 63°C. Similarly, Bhaskaran *et al*. (2010), Aarthi *et al*. (2010) and Hamidinejat *et al*. (2010) observed 278 bp product using same set of primers for amplifying the ITS-1 sequence of *E. tenella*.

5.6.2.3 Eimeria necatrix

The species specific primers targeting ITS-1 region of *E. necatrix* amplified a 381 bp product at an optimum annealing temperature of 62°C. The same set of primers were used by Aarthi *et al*. (2010), Bhaskaran *et al*. (2010) and Kumar *et al*. (2014) in their studies yielding similar results.

5.6.2.4 Eimeria maxima

PCR was standardised for *E. maxima* using species specific primers targeting ITS-1 region and 145 bp product was amplified at an annealing temperature of 63.7°C. This was in accordance with the reports of Lew *et al*. (2003) and Aarthi *et al*. (2010) who reported a 145 bp product using the same primers.

Sixty three morphologically positive samples were subjected to PCR targeting ITS-1 region for molecular confirmation of the *Eimeria* spp. Out of 63 samples, 60 yielded positive PCR results of which, 29 (48.33 per cent) were positive for *E. tenella*, 23 (38.33 per cent) for *E. necatrix* and eight (13.33 per cent) for *E. maxima*. Aarthi *et al*. (2010) reported *E. tenella*, *E. necatrix* and *E. maxima* as the prevalent species in broilers in Tamil Nadu by nested PCR targeting the ITS-1 region. Similarly, Bhaskaran *et al*. (2010) reported highest incidence of *E. tenella* followed by *E. mitis*, *E. acervulina*, *E.brunetti* and *E. maxima* from commercial poultry farms in India using ITS-1 PCR. The results were also in agreement with the findings of Hamidinejat *et al*. (2010) who reported the prevalence of *E. tenella*, *E. necatrix*, *E. acervulina*, *E. mitis* and *E.*

maxima in poultry farms of Khuzestan by molecular examination using species specific PCR. The three samples, which could not be confirmed by PCR in this study, might have very low concentration of the oocysts which yielded low quantity DNA. Carvalho *et al.* (2011) had observed that samples containing at least 20 oocysts of each *Eimeria* spp. were necessary for PCR amplification. Similarly, Haug *et al*. (2007) explained that partial breakage of oocysts wall compromised the DNA yield, thereby reducing the sensitivity of PCR.

None of the samples yielded positive results with the species specific primers of *E. acervulina*, *E. brunetti*, *E. mitis* and *E. praecox* thus confirming the absence of these *Eimeria* species in the study area. Molecular confirmation of *Eimeria* spp. was done on 26 tissue samples obtained from the site of lesions (caeca and mid-intestines) in chicken by PCR. Of the 26 samples, 14 were positive for *E. tenella* and 12 for *E. necatrix*, which again confirmed the results of morphological examination.

5.6.3 Polymerase Chain Reaction for Detection of *Eimeria* spp. in Quail

The PCR protocol was standardised using the universal primers targeting ITS-1 region of *Eimeria* genus. A product of approximately 600 bp was amplified at an optimum annealing temperature of 57.3°C.

5.7 NUCLEOTIDE SEQUENCE ANALYSIS

Column purification and custom bidirectional sequencing was done for the PCR amplicons specific for *E. tenella*, *E. necatrix* and *E. maxima*. The sequences on BLAST analysis showed 100 per cent similarity with other published sequences of *E. tenella* and 99 per cent similarity with other published sequences of *E. necatrix* and *E.maxima* isolates in Gen Bank. The partial ITS-1 sequences of *E. tenella*, *E. necatrix* and *E.maxima* were submitted to Gen Bank through BankIt and accession numbers were obtained. Similarly, the studies of Lew *et al.*, (2003), Bhaskaran *et al*. (2010) and Kumar *et al*. (2014) reported ITS-1 sequences of *E. tenella*, *E. necatrix* and *E.maxima*.

The sequence of *Eimeria* spp. in quail on BLAST analysis showed 95 per

cent similarity with the other published sequences of ITS-1 region of *Eimeria* spp.

5.8 PHYLOGENETIC ANALYSIS

Phylogenetic analysis was carried out for ITS-1 sequences of *E. tenella*, *E. necatrix* and *E.maxima* using 'MEGA X' programme of Lasergene software using maximum likelihood method based on General Time Reversible model with 1000 bootstrap value. The corresponding gene sequences of *Toxoplasma gondii* was used as outgroup for analysis. On phylogenetic analysis, *E. tenella* Kerala isolate was found to be evolutionarily related to the isolates of Mathura, Bareily, Tamil Nadu and Andra Pradesh and was distinct from the isolate of China. The Kerala isolate of *E. necatrix* occurred as a distinct clade from other isolates of China, UK, Australia and Faizabad while the Kerala isolate of *E. maxima* occurred in same clade with isolates of Australia, Tamil Nadu and China and was in a sister clade to the isolate of Faizabad.

From the present study it could be concluded that the conventional identification of *Eimeria* spp. based on oocysts morphology, sporulation time and pathological lesions have limitations due to their overlapping characteristics among the different species. The results of the study demonstrate that the molecular identification by ITS-1 based PCR allows species specific detection of *Eimeria* in chicken. We could not encounter any previous reports on molecular identification of *Eimeria* spp. in Kerala and this forms the first report on molecular detection of *E. tenella*, *E. necatrix* and *E. maxima* in chicken in Kerala. To the best of our knowledge, this also forms the first report of identification of *Eimeria* spp. in quail in Kerala. The partial ITS-1 sequences of *Eimeria* spp. in quail were sequenced and submitted to Gen Bank. *Eimeria* spp. infection could not be identified in any of the samples from turkeys during the study period which may be attributed to the limited number of samples examined due to the lesser turkey population in the study area.

Summary

6. SUMMARY

- Avian coccidiosis is an economically important parasitic disease of poultry. The etiological causes of coccidiosis are several apicomplexan parasites of the genus *Eimeria* that infect the gut and are transmitted between birds via ingestion of infective oocysts. The seven *Eimeria* spp. infecting chicken are *E. tenella*, *E. necatrix*, *E. maxima*, *E. acervulina*, *E. mitis*, *E. brunetti* and *E. praecox*. The *Eimeria* spp. that have been reported in quail are *E. bateri*, *E. tsunodai* and *E. uzura* while the species of *Eimeria* in turkey are *E. meleagridis*, *E. dispersa*, *E. gallopavonis*, *E. meleagrimitis* and *E. innocua*. Identification of *Eimeria* spp. in chicken, turkey and quail has important implications in studying the epidemiology as well as management of the disease. The aim of present study was to study the occurrence of *Eimeria* spp. infecting chicken, turkey and quail reared in different management systems and the species confirmation of *Eimeria* spp. in chicken by PCR.
- The occurrence of *Eimeria* spp. in chicken was studied by examining a total of 300 faecal samples, of which, 167 were from organised poultry farms and 133 from backyard poultry units, collected from areas in and around Thrissur district during the period from June 2019 to December 2020. Faecal samples were also collected from 150 quails and 50 turkeys reared in organised farms from the study area. Twenty-six intestinal samples from chicken suspected of coccidiosis and two samples from quail were collected from post mortem cases from the department of Veterinary Pathology, College of Veterinary and Animal Sciences, Mannuthy.
- The overall occurrence of *Eimeria* spp. in chicken was 37.66 per cent (113/300) and in quail 16.66 per cent (25/150). None of the turkey samples found to be positive for coccidia. Morphological identification of *Eimeria* spp. in chicken and quail was done based on the sporulation time, shape of the oocysts, presence/absence of the micropyle and morphometry. Three species of *Eimeria* in chicken *viz. E. tenella, E. necatrix* and *E. maxima* and two species of *Eimeria* in quail, *E. bateri* and *E. tsunodai,* were

identified based on morphological features. Oocysts of *E. tenella* and *E. necatrix* were ovoid in shape whereas the oocysts of *E. maxima* were broadly ovoid. The average micrometric values of oocysts of *E. tenella*, *E. necatrix* and *E. maxima* were 21 X 18.25 µm, 23.77 X 22.05 µm and 28 X 22.5 µm with shape index of 1.15, 1.07 and 1.24, respectively. The average size of *E. bateri* and *E. tsunodai* oocysts were 23 X 15.4 µm and 20 X 14.5 µm with shape index of 1.49 and 1.37, respectively.

- On morphological identification *E. tenella* was found to be the most predominant species in chicken with occurrence rate of 46.01 per cent which was significantly higher compared to that of *E. necatrix* (39.82 %) and *E. maxima* (14.15 %). The rate of occurrence of *Eimeria* infection was found to be significantly higher in backyard poultry (45.86 %) compared to that in organised farms (31.13 %). There was no significant difference between the occurrence rates in birds of different age groups. The highest occurrence was found to be during monsoon (43.13 %) which decreased to 41.42 per cent during post-monsoon and 18.96 per cent in pre-monsoon.
- Out of 26 intestinal samples of coccidiosis suspected chicken, 14 samples were positive for caecal coccidiosis and 12 for intestinal coccidiosis. The gross pathological lesions showed ballooning of intestines (jejunum) with petechial hemorrhages and thickened intestinal wall. The scrapings from the intestine revealed oocysts of *E. necatrix* and *E. maxima*. The observed average lesion score was 3+. The gross pathological lesions of caeca included petechial haemorrhages, distended caeca filled with blood and thickened caecal walls. The caecal scrapings revealed oocysts of *E. tenella* and the observed average lesion score was 2+. The gross pathological lesions in the two quail intestinal samples included slight ballooning of the intestine and caeca, congested mucosa, numerous petechiae on the serosal surface with noticeable amount of blood in the contents. The average lesion score for intestine and caeca in quail was 2+.
- The histopathology of jejunum showed marked destruction of villi, desquamation of epithelial cells, presence of schizonts in the intestinal

epithelium and various developmental stages of coccidia in the border cells of villi. The histopathological section of caecum revealed sloughing and destruction of villi with schizonts and the immature oocysts in the epithelial cells. The caecal smears showed presence of schizonts and merozoites. The schizonts were oval in shape filled with merozoites, and the merozoites were found to be elliptical. The histopathological section of intestine and caeca of quail revealed desquamation of epithelium of intestine and the presence of various developmental stages of coccidia in caeca.

- The genomic DNA was isolated from the sporulated oocysts of *Eimeria* spp. and tissue samples by using phenol chloroform method and commercial DNA extraction kit, respectively. The PCR protocol was standardised by gradient PCR using primers targeting the ITS-1 region of the seven *Eimeria* spp. in chicken. A 750 bp product was amplified by PCR using primers targeting ITS-1 region of *Eimeria* genus. Species specific primers targeting ITS-1 region of *E. tenella, E. necatrix* and *E. maxima* yielded 278 bp, 381 bp and 145 bp products, respectively. The PCR with other four species specific primers *viz*. *E. acervulina*, *E. brunetti*, *E. mitis* and *E. praecox* yielded no detectable amplified products, thus confirming the absence of these species in the samples studied.
- The amplicons of *E. tenella*, *E. necatrix* and *E. maxima* were sequenced and the partial ITS-1 sequences were submitted to Gen Bank using Bank It and accession numbers were obtained. Phylogenetic analyses for these three species was carried out using 'MEGA X' programme of Lasergene software using maximum likelihood method based on General Time Reversible model and analysed with 1000 bootstrap value.
- Sixty-three morphologically positive samples were subjected to PCR targeting ITS-1 region for molecular confirmation of the *Eimeria* spp. Out of 63 samples, 60 yielded positive PCR results of which, 29 (48.33 %) were positive for *E. tenella*, 23 (38.33 %) for *E. necatrix* and eight (13.33 %) for *E. maxima*.

- From this study it could be concluded that the conventional identification of *Eimeria* spp. based on oocysts morphology, sporulation time and pathological lesions have serious limitations due to their overlapping characteristics among the different species. The results of the present study demonstrate that the molecular identification by ITS-1 based PCR allows species-specific detection of *Eimeria* spp. This forms the first report on molecular detection of *E. tenella*, *E. necatrix* and *E. maxima* in chicken in Kerala. To the best of our knowledge, this also forms the first report of identification of *Eimeria* spp. in quail in Kerala.

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OCCURRENCE AND IDENTIFICATION OF *EIMERIA* SPECIES IN CHICKEN, TURKEY AND QUAIL

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(18-MVM-47)

ABSTRACT OF THESIS

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MASTER OF VETERINARY SCIENCE

(Veterinary Parasitology)

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Faculty of Veterinary and Animal Sciences

Kerala Veterinary and Animal Sciences University

DEPARTMENT OF VETERINARY PARASITOLOGY

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ABSTRACT

The study was undertaken during the period from June 2019 to December 2020 with the objectives of studying the occurrence of *Eimeria* spp. in chicken, turkey and quail in organised poultry farms and backyard poultry units by conventional methods and molecular confirmation of *Eimeria* spp. in chicken by polymerase chain reaction (PCR). A total of 300 chicken, 150 quail and 50 turkey faecal samples collected from organised poultry farms and backyard poultry units in and around Thrissur district were examined by conventional methods for morphological identification of *Eimeria* species. Twenty-six intestinal samples of coccidiosis suspected chicken and two samples from quail were also collected from post mortem cases from the department of Veterinary Pathology, College of Veterinary and Animal Sciences, Mannuthy. The overall occurrence of *Eimeria* spp. was 37.66 per cent (113/300) in chicken and 16.66 per cent (25/150) in quails. None of the samples collected from turkeys were found to be positive for *Eimeria* spp. The species of *Eimeria* identified on morphological examination were *E. tenella*, *E. necatrix* and *E. maxima* in chicken and *E. bateri* and *E. tsunodai* in quail. The occurrence rate of *E. tenella* was found to be significantly higher (46.01 %) compared to *E. necatrix* (39.82 %) and *E. maxima* (14.15 %). The rate of occurrence of *Eimeria* infection was found to be significantly higher in backyard poultry (45.86 %) and during the monsoon season (43.13 %). Gross lesions found in the intestine and caeca of chicken included ballooning of intestine with petechial haemorrhages, distended caeca filled with blood and thickened caecal and intestinal walls, with average lesion score of 3+ and 2+, respectively. In quail the gross lesions included ballooning of the intestine and caeca with blood in the contents and the average lesion score was 2+. The histopathological examination of intestinal and caecal lesions in chicken revealed marked destruction of villi with schizonts and the immature oocysts in the epithelial cells. In quail the histopathological examination of intestine and caeca revealed desquamation of epithelium, destruction of villi and presence of various developmental stages of *Eimeria*. The genomic DNA was isolated from the

sporulated oocysts of *Eimeria* spp. and tissue samples. The PCR protocol was standardised by gradient PCR using primers targeting the ITS-1 region of the seven *Eimeria* spp. in chicken. Out of 63 samples confirmed by conventional methods, 60 samples yielded PCR results of which, 29 (48.33 %) were positive for *E. tenella*, 23 (38.33 %) for *E. necatrix* and eight (13.33 %) for *E. maxima*. The amplicons of *E. tenella*, *E. necatrix* and *E. maxima* were sequenced and submitted to Gen Bank and the phylogenetic analysis was carried out. Partial ITS-1 region of *Eimeria* spp. in quail was amplified using universal primers, sequenced and submitted to Gen Bank. This study forms the first report of molecular identification of *Eimeria* spp. in chicken in Kerala. To the best of our knowledge, this also forms the first report of identification of *Eimeria* spp. in quail in Kerala.

Acad. Form No. 19

KERALA VETERINARY AND ANIMAL SCIENCES UNIVERSITY

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PROGRAMME OF RESEARCH WORK FOR THESIS FOR MASTERS DEGREE

1. **Title of thesis:**

   Occurrence and identification of *Eimeria* species in chicken, turkey and quail

2. **(a) Title of the departmental/KVASU research project of which this forms a part:** Nil

   (b) Code No. if any, and order by which the departmental/KVASU research project is approved: Nil

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   College of Veterinary and Animal Sciences, Mannuthy, Thrissur- 680 651

5. **Objectives of the study:**

   1. Study the occurrence of *Eimeria* spp. in chicken, turkey and quail in organised poultry farms and backyard poultry units by conventional methods
   2. Molecular confirmation of *Eimeria* spp. in chicken by polymerase chain reaction (PCR)

6. **Practical/Scientific utility:**

   Coccidiosis, a disease caused by the genus *Eimeria*, has a significant economic impact on poultry production. Conventionally, identification of *Eimeria* spp. is based on morphological features of the sporulated oocyst, sporulation time and location/scoring of pathological lesions in the intestine but the procedures involved require specialist expertise and have serious limitations due to their subjective nature and overlapping characteristics among different species.

   The development of molecular tools has made easy not only the diagnosis, but also the study on genetic variability of pathogens based on small quantity of oocysts through species-specific molecular markers.

The study on the occurrence of *Eimeria* spp. in other gallinaceous birds likes turkey and quails have not been undertaken in Kerala so far. Hence, the present topic is proposed to study the occurrence of *Eimeria* spp. in chicken, turkey and quails and for molecular confirmation of the species in chicken by PCR.

7. Important publications on which the study is based:

Teixeira *et al.* (2004) identified three species of *Eimeria viz., E. tsunodai, E. uzura* and *E. bateri* in Japanese quails in Brazil using morphological criteria and histopathological findings.

Haug *et al.* (2007) evaluated different methods of DNA extraction and PCR assays for identification of *Eimeria* spp. It was concluded that rupture of oocysts by minipestle grinding, extraction of DNA with GeneReleaser, followed by optimised single species PCR assays targeting species-specific internal transcribed spacer-1 (ITS-1) sequences was a sensitive protocol for identifying *Eimeria* spp. from field samples.

Aarthi *et al.* (2010) analysed the molecular prevalence of seven species of *Eimeria* in chicken in Tamil Nadu by nested PCR targeting the ITS-1 region and reported *E. necatrix, E. tenella* and *E. maxima* to be the prevalent species in broilers, while in layers the prevalent species were *E. necatrix, E. brunetti* and *E. maxima.*

Bhaskaran *et al.* (2010) reported the presence of multiple infections of *Eimeria* spp. with highest incidence of *E. tenella* followed by *E. mitis, E. acervulina, E.brunetti* and *E. maxima* from commercial poultry farms in India using ITS-1 PCR.

Carvalho *et al.* (2011) used a combination of morphological, pathological and molecular markers for identifying multiple circulating species of *Eimeria* in broiler flocks. Samples containing at least 20 oocysts of each *Eimeria* spp. were found to be necessary for PCR amplification. It was concluded that PCR was suitable as a routine diagnostic technique for monitoring the fluctuations in field Eimeria populations.

Sharma *et al.* (2013) recorded a higher prevalence rate for coccidiosis in backyard poultry (53.61%) when compared to organised farms (25.55 %) in Jammu.

The presence of multiple copies of genomic targets and use of nested PCR strategy made ITS-1 based nested PCR more sensitive than multiplex PCR based on sequence characterized amplified region (SCAR) markers in detection of *Eimeria* spp. in poultry (Kumar *et al.*, 2014).

Vrba and Pakandl (2014) characterized five species of *Eimeria* in turkeys by traditional and molecular methods involving sequencing of 18S rDNA, COI and ITS-1 regions and phylogenetic analysis. The species identified were *E. meleagridis, E. dispersa, E. gallopavonis, E. meleagrimitis* and *E. innocua.*

Prakashbabu *et al.* (2017) studied the occurrence of *Eimeria* spp. across different production systems using PCR and reported considerable diversity in the distribution of *Eimeria* spp. according to poultry unit size, system and management.

8. Outline of the technical programme:

Faecal samples will be collected from chicken, turkey and quails from organised poultry farms and backyard poultry units in and around Thrissur district. A minimum of 300 chicken, 150 quail and 50 turkey faecal samples will be collected. Age group and vaccination status of the birds will be recorded during collection of samples. Intestinal contents and scrapings will also be collected from postmortem cases suspected for coccidiosis from the Department of Veterinary Pathology, CVAS, Mannuthy. Samples will be confirmed for the presence of coccidial oocysts by microscopic examination.

Morphological and morphometric identification of oocysts will be done (Soulsby, 1982).

Gross and histopathological lesions in the intestine of affected birds will be studied (Bancroft and Suvarna, 2019).

Samples containing oocysts will be processed and DNA will be isolated using commercially available kit. Isolated DNA will be subjected to PCR to amplify ITS-1 region of *Eimeria* spp. detected in chicken for species identification (Haug *et al.*, 2007). The amplicons will be sequenced.

Phylogenetic analysis of ITS-1 sequence of different *Eimeria* spp. in chicken will be carried out.

Data will be statistically analysed (Snedecor and Cochran, 1994).

9. Main items of observations to be made:

1. Oocyst morphology of *Eimeria* spp. in chicken, turkey and quails
2. Gross and histopathological findings of intestine
3. Specific PCR amplicons of individual *Eimeria* spp. in chicken
4. Sequence data
5. Phylogenetic tree

10. Facilities

(a)Existing:

Work will be commenced with existing facilities available in the Department of Veterinary Parasitology, other departments and Central Instruments Laboratory of College of Veterinary and Animal Sciences, Mannuthy.

(b) Additional facilities required:

Chemicals and biologicals

11. Duration of study:

Four semesters

12. Financial estimate:

Cost of chemicals and Biologicals	: Rs.20, 000
Contingencies	: Rs.5, 000
Total	: Rs. 25,000

Signature of student

Project coordination group to which the proposal is to be placed:

Avian diseases

Signature of Major Advisor

Mannuthy,

22.06.2019

Name and signature of members of the Advisory Committee

1. Dr. Asha Rajagopal
   Assistant Professor
2. Dr. K.Devada
   Professor and Head
3. Dr. Priya M.N.
   Assistant Professor
4. Dr. I.S. Sajitha
   Assistant Professor

APPENDIX-I

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APPENDIX II

Time frame of work

Semester I

1. Collection of literature
2. Preparation of research proposal

Semester II

1. Collection of literature continued
2. Collection of samples from farms / backyard flocks and postmortem

Semester III

1. Collection of samples continued
2. Processing of samples, morphological identification and histopathological studies
3. DNA isolation and standardisation of PCR

for detection of *Eimeria* spp.

Semester IV

1. Morphological identification and molecular confirmation of *Eimeria* spp. will be continued
2. Analysis and interpretation of the results and submission of thesis

CERTIFICATE

Certified that the research project has been formulated observing the stipulations laid down under the Prevention of Cruelty to Animals Act (Amendment, 1998)

Place: Mannuthy
Date: 22-06-2019

Dr. Asha Rajagopal
Major Advisor

CURRICULUM VITAE

Name of candidate	: Dr. Pooja G Mankani
Date of birth	: 07.07.1995
Place of birth	: Vijayapura
Marital status	: Unmarried
Permanent address	: At.Post- Nidagundi, Taluka- Nidagundi, District- Vijayapura
Major field of specialization	: Veterinary Parasitology
Educational status	: BVSc & AH, undergoing MVSc
Publications made	: Nil
Accepted article for publication	: Nil
Membership in professional societies	: 1. Karnataka State Veterinary Council 2. Indian Veterinary Association 3. Indian Association for the Advancement of Veterinary Parasitology

OCCURRENCE AND IDENTIFICATION OF *EIMERIA* SPECIES IN CHICKEN, TURKEY AND QUAIL

POOJA G. MANKANI

(18-MVM-47)

ABSTRACT OF THESIS
(MALAYALAM)

Submitted in the partial fulfillment of the requirement for the degree of

MASTER OF VETERINARY SCIENCE

(Veterinary Parasitology)

2021

Faculty of Veterinary and Animal Sciences

Kerala Veterinary and Animal Sciences University

DEPARTMENT OF VETERINARY PARASITOLOGY

COLLEGE OF VETRINARY AND ANIMAL SCIENCES

MANNUTHY, THRISSUR 680651

KERALA, INDIA

സംഗ്രഹം

കോഴി, ടർക്കി, കാട എന്നിവയിൽ ഐമീരിയ ഇനങ്ങളുടെ വ്യാപനം

കോഴി, ടർക്കി, കാട എന്നിവയിൽ രക്താതിസാരത്തിനു കാരണമായ ഐമീരിയയുടെ വ്യാപനം പഠിക്കുക എന്ന ലക്ഷ്യത്തോടെയാണ് ഈ പഠനം നടത്തിയത്. തൃശ്ശൂർ ജില്ലയിലും പരിസരത്തുമുള്ള സംഘടിത കോഴി ഫാമുകളിൽ നിന്നും വീട്ടു മുറ്റത്തെ കോഴി യൂണിറ്റുകളിൽ നിന്നും ശേഖരിച്ച 300 കോഴി, 150 കാട, 50 ടർക്കി എന്നിവയുടെ കാഷ്ഠ സാമ്പിളുകളിൽ കാഷ്ഠ പരിശോധന, സ്പോറുലേഷൻ സമയം തുടങ്ങിയ പരമ്പരാഗത രീതികൾ വഴി ഐമീരിയയുടെ ഇനം തരംതിരിച്ചു. ഇതു കൂടാതെ രക്താതിസാരം സംശയിക്കപ്പെട്ട കോഴികളിൽ നിന്നും, കാടകളിൽ നിന്നും പോസ്റ്റ്മോർട്ടം സാമ്പിളുകൾ ശേഖരിച്ച് അവയിലെ ക്ഷതികങ്ങളുടെ പഠനം നടത്തുകയു □ ായി. കോഴികളിൽ ഐമീരിയയുടെ വ്യാപനം 37.66 ശതമാനവും കാടകളിൽ 16.6 ശതമാനവും ആണെന്നു ക□ ത്താനായി. ടർക്കികളിൽ നിന്നു ശേഖരിച്ച സാമ്പിളുകളിലൊന്നും ഐമീരിയ ബാധ ക□ ത്തിയില്ല. കോഴികളിൽ ഐമീരിയ ടെനെല്ല, ഇ.നിക്കാട്രിക്സ്, ഇ.മാക്സിമ എന്നീ മൂന്നു ഇനങ്ങളും, കാടകളിൽ ഇ.ബാറ്റേരി, ഇ.സുനോഡായ് എന്നീ ഇനങ്ങളും രൂപാന്തര പരിശോധനയിലൂടെ ക□ ത്തി. കൂടാതെ, വിട്ടുമുറ്റത്തെ കോഴി യൂണിറ്റുകളിലും, മഴക്കാലത്തും ഐമീരിയയുടെ വ്യാപനം വളരെ കൂടുതലാണെന്നും ഈ പഠനം വഴി ക□ ത്താനായി.

ഐമീരിയ ബാധമൂലം കുടലുകളിലും, സീക്കത്തിലുമു□ ാകുന്ന ക്ഷതികങ്ങളുടെ തീവ്രത യഥാക്രമം 3+ ഉം 2+ ഉം ആയിരുന്നു. കുടലിന്റെ ബലൂണിംഗ്, രക്തസ്രാവം, കുടൽ ഭിത്തികൾ കട്ടിയാകൽ എന്നീ ക്ഷതികങ്ങൾ പൊതുവായി കാണപ്പെട്ടു. ഹിസ്റ്റോപത്തോളജി പരിശോധനയിൽ കുടലിലെ വില്ലകളുടെ നാശം, എപ്പീത്തിലിയിൽ കോശങ്ങളുടെ അപചയം, ഐമീരിയ ഊസിസ്റ്റുകളുടെ സാന്നിധ്യം എന്നിവ ക□ ത്താനായി

കോഴികളിലെ ഐമീരിയ ഊസിസ്റ്റുകളിൽ നിന്നും വേർതിരിച്ചെടുത്ത ഡി.എൻ.എ. ഉപയോഗിച്ച് പി.സി.ആർ. പരിശോധന വഴി തന്മാത്രാ സ്ഥിരീകരണവും നടത്തുകയു□ ായി. ഏഴ് ഐമീരിയ ഇനങ്ങളിലെ ഐ.ടി.എസ്. മേഖല

അടിസ്ഥാനമാക്കുന്ന പ്രൈമറുകൾ ഉപയോഗിച്ചാണ്. പി.സി.ആർ. പരിശോധന നടത്തിയത്. പരമ്പരാഗത രീതികളാൽ സ്ഥിരീകരിച്ച 63 സാമ്പിളുകളിൽ 60 സാമ്പിളുകൾ പി.സി.ആർ. ഫലങ്ങൾ നൽകി. അതിൽ 29 (48.33%) ഇ.ടെനെല്ലക്കും 23 (38.33%) ഇ.നിക്കാട്രിക്സീനും, 8 (13.33%) ഇ.മാക്സിമക്കും പോസിറ്റീവ് ആയിരുന്നു. ഐമീരിയ ടെനെല്ല, ഇ.നിക്കാട്രിക്സ്, ഇ.മാക്സിമ എന്നീ ഇനങ്ങളുടെ ഭാഗിക ഐ.ടി.എസ്. മേഖലയുടെ ഫൈലോജനറ്റിക് വിശകലനം നടത്തുകയു□ ായി. സാർവത്രിക പ്രൈമറുകൾ ഉപയോഗിച്ച് കാടകളിൽ ഐമീരിയ ജനുസ്സിന്റെ തന്മാത്രാ സ്ഥിരീകരണം നടത്തി. കോഴികളിൽ കാണപ്പെടുന്ന ഐമീരിയ ഇനങ്ങളുടെ തന്മാത്രാ സ്ഥിരീകരണത്തിന്റെ കേരളത്തിലെ ആദ്യ റിപ്പോർട്ടാണ് ഈ പഠനം. കൂടാതെ, കേരളത്തിലെ കാടകളിൽ കാണപ്പെടുന്ന ഐമീരിയ ഇനങ്ങളെക്കുറിച്ചുള്ള ആദ്യ റിപ്പോർട്ടും ആകുന്നു.