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PARASITOLOGICAL,MOLECULAR AND HISTOPATHOLOGICAL STUDIES ON *E. tenella* INFECTING CHICKEN IN EGYPT

By

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ABSTRACT

Coccidiosis in chickens is one of the major problems of poultry industry that is caused by protozoan parasites of genus *Eimeria*. Cecal contents and cecal mucosal scraping samples were collected from naturally infected native broiler chicken flock aging 25 days. The post mortem examination showed hemorrhagic mucosa with bloody caecal core in two caeca. *Eimeria* oocysts were isolated by single oocyst technique. The obtained sporulated oocysts were identified according to morphological features, and the calculated average oocyst shape index of randomly 50 oocysts (1.22 µm), average length (25.2 µm) with SD (±2.24 µm), and average width (20.6 µm) with SD (±1.6µm) as *E. tenella*. Species-specific polymerase chain reaction (PCR) test targeting the internal transcribed spacer-1 (ITS-1) sequences of the genomic rDNA using primer pairs (*E. tenella ET*, *E. maxima Ema* and *Emu* and *E. nexatrix EN*) was performed for isolated *Eimeria* species**.** The results of species-specific PCR assays confirmed the presence *o*f *E. tenella* as it has the characteristic in all of the tested samples bp of 278 but there wasn't band with other previous mentioned primer pairs. Histopathological evidence showed leakage of blood, oedema and necrosis of caecal mucosa.*Eimeria tenella* developmental stages in caecum were alsodetected histopathologically.

Keywords:

Coccidiosis - *E. tenella* - single *oocyst* - PCR- broiler *chicken.*

INTRODUCTION

Coccidiosis is one of the most important diseases in commercial poultry production worldwide, caused by protozoan parasites of the genus *Eimeria* that cause tissue damage, resulting in interruption of feeding and digestive processes or nutrient absorption; dehydration; blood loss; and increased susceptibility to other disease agent **Morris and Gasser (2006)**. The infection is caused by one or several of the nine *Eimeria* species

(*E. tenella*, *E. acervulina, E. brunetti*, *E. maxima, E. mivati*, *E. mitis*, *E. necatrix*, *E. praecox* and *E. hagani*) which develop within the intestine of most wild and domestic birds leading to different forms of subclinical enteric infection to subacute mortality **McDougald and Fitz-Coy (2013).** These species have different properties regarding localization in the gut, pathogenicity, pre-patent period, fecundity and immunogenicity.

Caecal coccidiosis was at one time the commonest type of coccidiosis. It is caused by *Eimeria tenella* and results in lesions in the caecum of chicken's worldwide causing morbidity ranged from 10% to 40% and mortality up to 50% **McMullin, (2004).**

Eimeria tenella histopathological lesions are loss of epithelial tissue, congestion of blood vessels, which indicate disruption followed by leakage of blood and severe muscular oedema, necrosis of mucosa, submucosa of caecum, presence of clusters of oocysts and schizonts and lymphoid cells hyperplasia. Mononuclear cell infiltration was noted in the mucosal layer **patra *et al.*, (2010).**

Diagnosis of coccidiosis is based on several traditional methods include comparing clinical features, gut pathology as well as morphology of different parasite stages in faeces or intestinal content and pre-patent period **Long and Reid (1982)**. Recently, techniques based on using PCR amplification of DNA have been used for the diagnosis of *Eimeria* spp. in poultry. A number of approaches have proved to be both specific and highly sensitive for analyses either of parasites grown *in vitro* or present in tissue samples and clinical materials **Carvalho *et al.,* (2011).**

Accurate identification of *Eimeria* spp. is important not only for the diagnosis of disease but also for management of subclinical infection, development and application of effective control strategies, and biological and epidemiological study **Kumara *et al.,* (2014).**

The present study was planned to cover the following points:

1-Isolation and identification of the species parasitizing the cecum of chickens by single oocyst technique at Giza government.

2-Molecular characterization of the isolated oocyst of *E .tenella*.

3-Histopathological examination of the lesions caused by *E. tenella* in the cecum of chicken.

MATERIAL AND METHODS

Samples for isolation of *Eimeria* oocysts:

Field *Eimeria* species' oocysts were isolated from caecal contents and caecal mucosa of naturally infected native broiler chicken flock aging 25 days.

Isolation of *Eimeria* oocysts:

Eimeria oocysts were isolated by single oocyst technique according to the method of **Karim and Tress (1990)** and then kept for sporulation in 2.5% pot. Dichromate solution **Conway and McKenzie (2007)**.

Parasitologic examination:

Using a calibrated ocular micrometer at 400x magnification, saturated salt floatation technique was used to examine oocysts for length and width measurements. Fifty random oocysts from sample were examined by measuring their length and width using microscope with calibrate ocular lens as well as determination of the oocysts shape index (Length/Width) and morphology of oocysts **Conway and McKenzie (2007).**

Sample preparation for DNA extraction:

The purified oocysts, stored in 2.5% potassium dichromate solution, were washed 4 times by centrifugation in autoclaved phosphate buffered saline. The pellet contains oocyst concentration (50,000 oocysts) of isolate which was determined by haemocytometer (Neubauer Improved, Germany) was resuspended in 400 µl TE buffer and sonicated at high speed sonication using Bandelin Sonoplus Sonicator, GERMANY for 90 second **Patra *et al.*, (2010).**

DNA extraction:

DNA was extracted by Classic phenol-chloroform method. Extracted DNA concentration was measured by Thermo Scientific Nano Drop 2000 spectrophotometer (USA) **Sambrook and Russell (2001).**

***PCR* amplification**:

DNA amplification by conventional PCR assay:

PCR analysis was done by using primer pair *Ema*, *Emu*, *EN* and *ET* in standard PCR procedures. The primers identification, the amplified DNA fragments and primers'references were shown in the following table.

Table (1): Sequence primer pairs used in conventional PCR.

Species	Primer name	Primer Sequences 5′- 3′	PCR product size (bp)	Annealing temp./time	Reference
E. tenella	*ET* *ET*	F: 5′-AATTTAGTCCATCGCAACCCTTG-3′ R: 5′-CGAGCGCTCTGCATACGACA-3′	278	58°c/1min	Haug *et al.*, (2007)
E. maxima	*Emu* *Emu*	F: 5′-TTGTGGGGCATATTGTTGTGA-3′ R: 5′-CWCACCACTCACAATGAGGCAC-3′	162		
E. necatrix	*EN* *EN*	F: 5′-AGTATGGGCGTGAGCATGGAG-3′ R:5′GATCAGTCTCATCATAATTCTCGCG-3′	160		
E. maxima	*Ema* *Ema*	F: 5′-GTGGGACTGTGGTGATGGGG-3′ R: 5′-ACCAGCATGCGCTCACAACCC-3′	205		

Histopathology:

Intestinal tissues were fixed in formalin solution for 1-2 hour and washed it several changes of 50% alcohol for 4 - 6 hour and stored in 70 % alcohol then washed in 80%, 90%, 95% and absolute, finally the specimens were embedded in paraffin wax, sectioned and stained with H and E. stain **Urara *et al.*, (2005)**.

RESULTS

Natural infected broiler chicken flock showing, signs of depression, weakness, bloody diarrhoea, anorexia and ruffled feathers. Postmortem examination showed caecal lesions ranged from hemorrhages to present of caecal cores, debris with haemorrhages on the mucosa. The bloody mass in the caeca, others showed mottled reddish or milky white colored contents in the caeca due to formation of oocysts.

Direct cecal mucosal smear:

Direct cecal mucosal smear reveal to presence of unsporulated oocyts in the examined field.

Morphology and Measurements of oocysts:

Detected oocyst was ovoid with measurements indicated that obtained oocysts were *Eimeria tenella* as shown in (Table 2).The calculated average oocyst shape index of randomly 50 oocysts was (1.22 µm) as shown in (Table 3), average length (25.2 µm) with SD (±2.24 µm), average width (20.6 µm) with SD (±1.6 µm) Fig. (1).

Table (2): Measurements of 50 *E. tenella* oocysts size.

NO. of oocyst	Size – µm			NO. of oocyst	Size - µm		
	Length	Width	Shape Index		Length	Width	Shape Index
1	24.5	21	1.17	26	28	21	1.33
2	24.5	17.5	1.4	27	24.5	21	1.17
3	24.5	21	1.17	28	28	17.5	1.6
4	28	21	1.33	29	24.5	21	1.17
5	24.5	21	1.17	30	24.5	21	1.17
6	21	17.5	1.2	31	21	17.5	1.2
7	24.5	21	1.17	32	24.5	21	1.17
8	24.5	21	1.17	33	28	22.8	1.23
9	28	22.8	1.23	34	24.5	21	1.17
10	28	21	1.33	35	24.5	21	1.17
11	24.5	21	1.17	36	28	22.8	1.23
12	24.5	21	1.17	37	24.5	21	1.17
13	24.5	21	1.17	38	24.5	21	1.17
14	24.5	21	1.17	39	21	17.5	1.2
15	21	17.5	1.2	40	28	21	1.33
16	28	21	1.33	41	24.5	21	1.17
17	24.5	21	1.17	42	21	17.5	1.2
18	28	22.8	1.23	43	24.5	21	1.17
19	24.5	21	1.17	44	28	21	1.33
20	28	22.8	1.23	45	24.5	21	1.17
21	24.5	21	1.17	46	21	17.5	1.2
22	28	22.8	1.23	47	24.5	21	1.17
23	28	21	1.33	48	28	22.8	1.23
24	24.5	21	1.17	49	28	17.5	1.6
25	24.5	21	1.17	50	24.5	21	1.17

Table (3): Showing Min. value, Max. Value, Average and SD of *E. tenella* oocyst size.

Size	Length(µm)	Width(µm)	Shape Index(µm)
Minimum value	**21**	**17.5**	**1.17**
Maximum value	**28**	**22.8**	**1.6**
Average	**25.2**	**20.62**	**1.226**
Standard. dev.(SD)	**±2.24**	**±1.6**	**±0.098**

PCR result of *Eimeria tenella* strain:

The amplification of the ITS1 region using species specific primer pairs yielded a product of approximately 278 bp as shown Fig. (2). the primer pair for *E. tenella* exclusively amplified their target DNA and there was no cross-species amplification by heterologus primer pairs.

Histopathological lesions of isolated *E. tenella*:

All histopathological lesions found in caecum Fig. (3) and other parts of intestine with no lesions. The lesions were loss of epithelial tissue, congestion of blood vessels, which indicated disruption followed by leakage of blood. There were also found in this study severe muscular edema with congestion, necrosis of mucosa with goblet cells activation, inflammatory cells proliferation in submucosa of caecum, hyperplasia of mucosa layer, submucosal edema and presence of clusters of oocysts and schizonts.

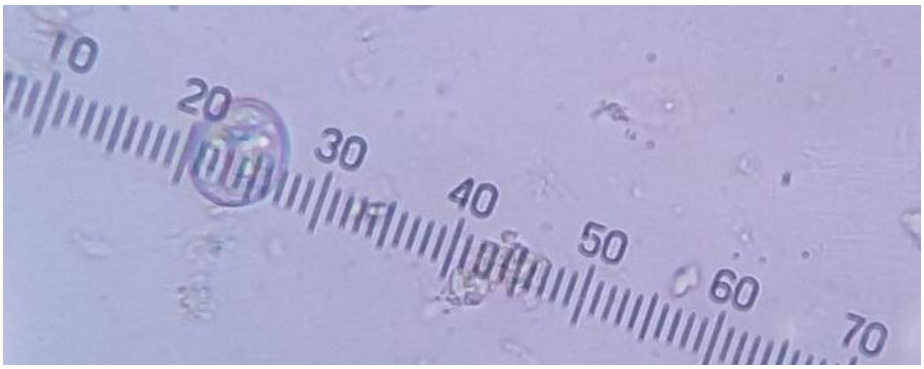

Fig. (1): Measured oocyst from single oocyst suspected to be *E. tenella* (X400)

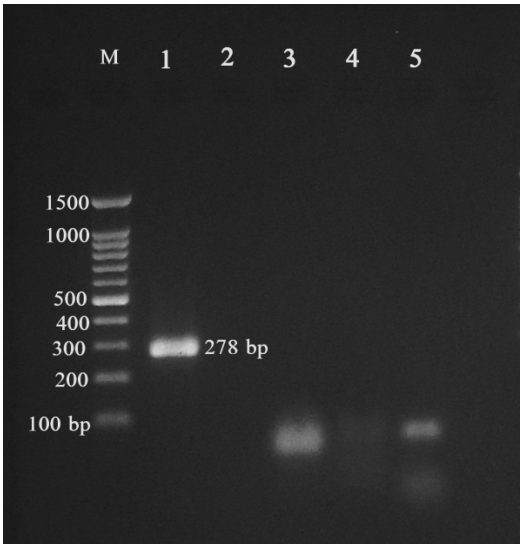

Fig. (2): Agrose gel electrophoresis. Polymerase Chain Reaction for *Eimeria tenella* strain
Lane M: 100 bp DNA ladder (Fermentas); Lane 1: *E. tenella*; Lane 2:*E. necatrix*; Lane 3: *E. maxima* a; Lane 4: *E. maxim*a b; Lane 5:Negative control

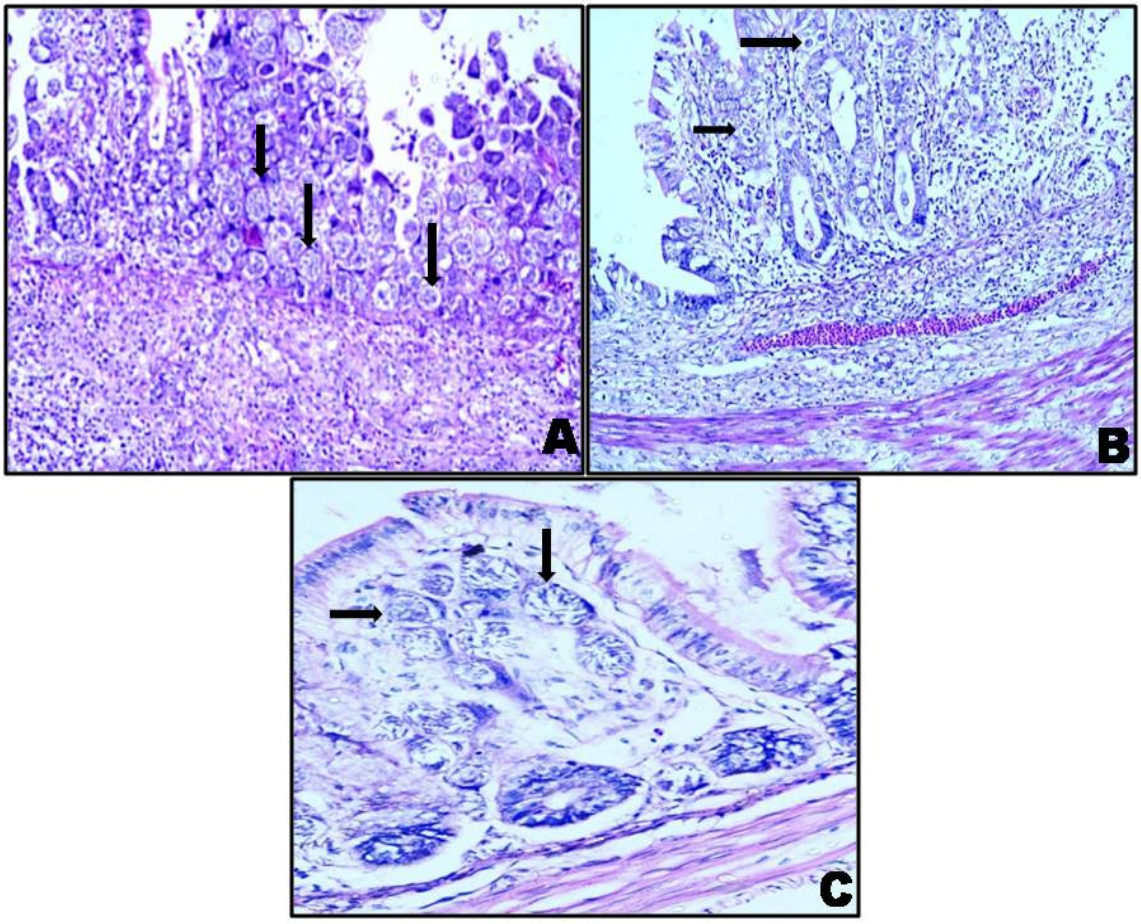

Fig. (3): Showing histopathological lesions of caecal section of infected chickens with *E. tenella* sporulated oocysts .

A. Section of caeca showing heavy developmental stages of schizonts (arrows) with severe epithelial necrosis. **(H&E. X200).**

B. Section of caeca showing moderate developmental stage of oocysts (arrows) with congested blood vessels. **(H&E. X200).**

C. Section of caeca showing severe developmental stages of schizonts (arrows) with severe edema. **(H&E. X 400).**

DISCUSSION

Coccidiosis is considered as one of the most economically important diseases of domestic poultry that is responsible for significant economic losses to the worldwide poultry industry. It remains one of the major diseases problems of poultry industry in spite of advances made in prevention and control through chemotherapy, management and nutrition **Siddiki *et al.*,**

(2014). *E. tenella* is the most pathogenic species due to morbidty, mortality, and greatly reduced weight gain **McDougald and Fitz-Coy (2013).**

In the present study, a simple method of single oocyst infection has been successfully applied to produce homogenous *Eimeria* isolates representing a single species from field samples, which contain mostly mixed *Eimeria* spp.

Our study detected that, the measurements of randomly 50 oocysts *E. tenella* were length (21-28µm) with average (25.2 µm), width (17.5-22µm) with average (20.6 µm) and shape index (1.22 µm). This result was near to the previous measurements recorded by **Olsen, (1986)** that measured the length of *E. tenella* oocysts to be (19.5 to 29µm) with average (26. 6µm) and (16.5 to 22.8µm) in width with an average 19 µm, **Amer *et al.,* (2010)** that his measurements were average length (21.39 µm), average width (18.745 µm) and shape index (1.24 µm) and **Kotpal, (2012)** mentioned that,the measurement were (20-26µm) by (16.5-23µm). Several PCR based assays targeting different regions of the *Eimeria* genome have been described, such as the 5S rRNA, the small subunit *rRNA* **Tsuji *et al.,* (1997)**, the sporozoite antigen gene EASZ 240/160 **Molloy *et al.,* (1998)** and ITS-1, first internal transcribed spacer **Schnitzler *et al.,* (1999)** and ITS-2 second internal transcribed spacer of ribosomal DNA **Lien *et al.,* (2007)** genomic regions. In the present investigation, interspecific DNA primers pair (*E. tenella* ET, *E .maxima Ema* and *Emu* and *E. nexatrix EN*) diversity within the ITS1 region of rDNA from our pathogenic species of *Eimeria* was examined. The *ET* primer pair examined was found to amplify fragment in a species specific manner that is consistent with the described previously by **Schnitzler *et al.,* (1998) and Yim *et al.,* (2011)**. In this study no cross amplification by the other three species specific primer were observed. Thus, the result of this study clearly demonstrates that, the PCR assay based on amplification of ITS1 regions of *Eimeria tenella* in poultry can be used for detection and identification of the parasite.

Histopathological lesions which were severe muscular edema with congestion, necrosis of mucosa with goblet cells activation, inflammatory cells proliferation in submucosa of caecum which found in the present investigation are similar to the findings of **Rasheda and Bano (1985) and Amer *et al.,* (2010)**.

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