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Research article

The effects of infectious dosages on endogenous phage and the excretion of *Eimeria tenella* oocysts of infected chicken

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Abstract

This study was conducted to evaluate the effects of four dosages of oocyst inoculation (1×10^2, 1×10^3, 1×10^4, and 1×10^5 oocysts/chick) on oocyst patterns in feces, lesion score, and the endogenous phase of *Eimeria tenella* in the tissue of infected chickens. Oocysts in feces were collected daily from 4 to 10 day-post-infection (dpi). Chickens were killed at 0, 3, 5, and 10 dpi to evaluate the lesion score and the endogenous phage in the tissue by hematoxylin and eosin staining. The results showed that chickens in the highest dosage group discharged oocysts the earliest (5 dpi), while oocysts were detected in the other 3 groups 1 day later (6 dpi). The results of the histopathological examination showed that the time to detect the development stages of *E. tenella* in the tissue was similar among the experimental groups. Specifically, the asexual stages (1st and 2nd schizonts) were detected at 3 dpi, and immature oocysts were found in the cecum tissue at 5 dpi in all experimental groups. Overall, the results indicated that the oocyst dosages do not affect the replication of *E. tenella* in intestinal epithelial cells but may influence the excretion of oocysts from the intestinal tissue.

Keywords: *Eimeria tenella*, dosages, chicken, oocyst

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INTRODUCTION

Coccidiosis is a common disease in poultry caused by a protozoan of the genus *Eimeria* (Conway and McKenzie, 2008). Seven species of *Eimeria* spp. were found in chickens (Joyner and Long, 1974). Chickens are infected with coccidiosis by ingesting sporulated oocysts through the gastrointestinal tract. The parasite then invades and undergoes intracellular reproduction in the intestinal epithelium. Chickens infected with coccidiosis have systemic symptoms such as anorexia, bloody diarrhea, weight loss, and death in chicks that are severely infected (Zaman et al., 2015). Of chickens infected with *Eimeria* spp., 20 % are clinical and 5 % are sub-clinical. Blake et al. (2020) reported that the global cost of coccidiosis in chickens is estimated to have been ~ £10.36 billion in 2016, including losses during production, costs for prophylaxis, and treatment.

Eimeria tenella is one of the most pathogenic protozoan parasites of the genus *Eimeria,* causing chicken coccidiosis worldwide (Quiroz-Castañeda, 2018). *E. tenella* infection is known to occur in the medial and distal parts of the chicken's cecum (Matsubayashi et al., 2012). The *E. tenella* life cycle consists of two phases of development: the exogenous phase in the environment and the endogenous phase in the cecal cells. The unsporulated oocysts in the feces of infected chickens undergo sporulation to acquire virulence in the exogenous phase. The sporozoites released from sporulated oocysts then start asexual reproduction (schizogony) in cecal cells, followed by sexual differentiation, fertilization, and the formation of unsporulated oocysts.

Understanding the factors that influence the developmental stages of *E. tenella* is valuable for controlling this disease in the future. Several studies reported that host immune responses contribute to the variations in parasite replication and disease severity (Lillehoj, 1988; Oakley et al., 2014; Boulton et al., 2018). Besides, some studies have shown that the severity of the disease also depends on infectious doses (Williams, 1973; Long et al., 1980). Chickens that ingest larger quantities of sporulated oocysts result in elevated parasite loads in the gut and increased oocyst outputs. However, the crowding effect is observed as fecundity is reduced when increasing dosage size (Williams, 2001). While several studies focused on the effects of parasite dosages on disease severity, there are very few reports on the effect of different infectious dosages on the developmental stages of *E. tenella* in cecal cells during infection. This research studies the impact of four *E. tenella* oocyst dosages by evaluating the oocyst pattern, lesion score, and the development of endogenous phase in the chicken cecum at 0, 3, 5, and 10 days after sporulated oocyst challenge.

MATERIALS AND METHODS

Ethics statement

All procedures were approved by the Animal Ethical Committee of Hue University (HUVN0022).

Animals

One-day-old broiler chickens (MD02) were purchased from Minh Du (Binh Dinh, Vietnam). All 1-day-old chicks received vaccinations against Marek's disease at the hatchery. Chicks were fed *ad libitum* with feed (corn, soybean, fish meal, rice bran, mineral premix, and multivitamins) processed to formulate diets that meet breeder requirements (crude protein 22 %, ME 3,100). The feed is free from antibiotics and anticoccidial drugs. Chick feces were collected and examined daily to confirm free oocysts before the start of the experiment, which will commence when they are 14 days old.

Parasites

The locally isolated virulent strain of *E. tenella* was maintained at the Laboratory of Parasitology, Hue University of Agriculture and Forestry (Hue, Vietnam), and the sixth passage of the strain was utilized. Purification of *E. tenella* oocysts was performed using the sugar flotation method, followed by sporulation at 28 °C in a 2.5 % potassium dichromate solution for 48 h (around 95 % of oocysts were sporulated), and stored at 4 °C for up to 1 month before use.

Experimental design

A total of 75 broiler chickens were allocated to five treatments with three replicates each. Each replicate consisted of five chickens housed in battery cages. The experiment had a completely randomized design. The treatments applied were as follows: Treatment 0 (T0), the negative control group, where 1 mL of phosphate-buffered solution was administered; Treatment 1 (T1), administration of 100 *E. tenella* sporulated oocysts; Treatment 2 (T2), administration of 1000 *E. tenella* sporulated oocysts; Treatment 3 (T3), administration of 10,000 *E. tenella* sporulated oocysts; and Treatment 4 (T4), administration of 100,000 *E. tenella* sporulated oocysts. The chicks were administered 1 mL of the inoculum orally through oral gavage. Abnormal feces were monitored and recorded daily throughout the experiment. Oocysts in feces were collected daily from 4 to 10 days post-infection (dpi). Chickens (3 chicks/group) were killed at 0, 2, 4, 5, and 10 dpi to evaluate the lesion score and the intracellular reproductive stages in the tissue by hematoxylin and eosin (H&E) staining.

Oocyst quantification

Between the 3rd and 10th days post oocyst inoculation, fecal samples were collected daily. To obtain each sample, all the fecal material within each cage was mixed together. Subsequently, random selection was used to allocate the mixed samples into three 15-mL centrifuge tubes, with approximately 2 g of fecal material per tube. These tubes were utilized for oocyst quantification. Oocysts per gram (opg) of feces were quantified by the fecal flotation method using a saturated sucrose solution, following the procedure described by Ho et al. (2021). In summary, the fecal samples (2 g per tube) were thoroughly mixed with 10 ml distilled water. The mixture was then centrifuged at 4,000 rpm for 10 min using a Thermo Fisher centrifuge. After centrifugation, the supernatant was discarded, and 10 ml of a saturated sucrose solution was added to the tubes. The contents were mixed well and centrifuged at 4,000 rpm for 10 min. The resulting supernatant was carefully transferred to other 15 ml centrifuge tubes and thoroughly mixed.

To quantify the oocysts, a drop of the supernatant (10 µl) was placed on a glass slide, covered with a cover glass, and examined using light microscopy. The oocysts were counted three times per tube. The calculation for determining the opg of feces was performed as follows: opg = n × 500, where n represents the average number of counted oocysts.

Lesion score

Ceca lesion scores were estimated at 4 levels, as described by Joyce and Malcolm (1970). Briefly, the gross lesions were evaluated and classified as follows: level 0, no gross lesions were observed; level 1, only a few scattered petechiae were found on the cecal wall, with no thickening of the cecal walls and normal cecal contents; level 2, lesions were more numerous, accompanied by noticeable blood in the cecal contents. The cecal wall showed some thickening, and normal cecal contents were present; level 3, there were significant amounts of blood or cecal cores present. The cecal walls exhibited substantial thickening, and there were minimal to no fecal contents in the ceca; and level 4, the cecal wall showed severe distension with either blood or large caseous cores. Fecal debris was either absent or included within the cores. Additionally, dead birds were assigned a score of +4.

Histopathological observations

In this study, the middle part of the ceca, which had been fixed in formaldehyde, was extracted and embedded in paraffin. Subsequently, sections were cut at a thickness of 6 µm and then deparaffinized. Three chicks from each group were selected for this process. The sectioned specimens were stained with H&E. A total of six specimens per chick, with 200-µm intervals between them, were prepared for observation under light microscopy. The evaluation aimed to assess the level of parasite burden, including both asexual and sexual stages. Each H&E-stained specimen was examined in eight fields at a magnification of 200X. The parasite burden levels in the H&E sections were estimated microscopically. The results were recorded using the following scale: "+", indicating 1–10 clusters in the infected cecum section; "++", representing 10–30 clusters; and "+++", indicating >30 clusters. To ensure an unbiased evaluation, the histological slides were assessed by two independent researchers who possessed technical expertise in histopathology. The evaluation was conducted under blinded conditions, meaning that the researchers were unaware of the treatment groups while evaluating the slides.

Statistical analysis

The data were presented as the mean ± standard deviation. Statistical analysis was performed using the unpaired t-test with KyPlot Statistics (version 5.0) software.

RESULTS

Fecal oocyst shedding

The result of daily fecal oocyst shedding from 3–10 dpi is presented in Figure 1. No oocyst was detected in the feces of the T0 group throughout the experiment. The number of oocysts excreted in the feces of all infected groups was proportional to the infectious doses. The average number of opg feces 3–10 dpi was lowest in the T1 group and highest in the T4 group. Oocyst shedding commenced at 5 dpi in the T4 group, while in the T1, T2, and T3 groups, it began at 6 dpi. The maximum number of fecal oocysts was detected at 6 dpi for the T4 group ($1.1 \times 10^6 \pm 9.5 \times 10^4$ opg feces). The other 3 groups showed maximum numbers of fecal oocysts at 7 dpi, $4.5 \times 10^5 \pm 3.2 \times 10^4$ opg feces for the T1 group, $5.8 \times 10^5 \pm 3.2 \times 10^4$ opg feces for the T2 group, and $9.4 \times 10^5 \pm 1.3 \times 10^5$ opg feces for the T3 group (Figure 1). Significantly higher numbers of fecal oocysts were observed in the T4 group compared with the other groups at 5–9 dpi ($P<0.001$).

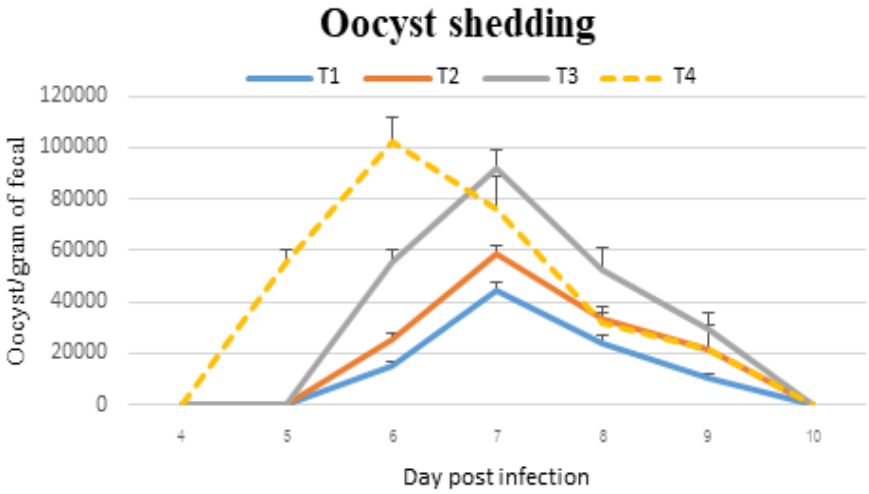

Figure 1 Fecal oocyst shedding of broiler chickens infected with different dosages of *Eimeria tenella*. Fecal oocyst shedding was measured in the T1 (treatment 1; challenged with 100 sporulated oocysts of *E. tenella*); T2 (treatment 2; challenged with 1,000 sporulated oocysts of *E. tenella*); T3 (treatment 3; challenged with 10,000 sporulated oocysts of *E. tenella*); T4 (treatment 4; challenged with 100,000 sporulated oocysts of *E. tenella*). Error bars represent standard deviation (SD).

Lesion score

The lesions score results on days 0, 3, 5, and 10 dpi showed that in all infected groups, lesions began to appear at 3 dpi, were most severe at 5 dpi, and recovered at 10 dpi. The extent of damage depends on the dose of infection. Chickens in the T0 group showed no gross lesions, those in the T4 group showed the highest score at 5 dpi (4 ± 0), and it gradually reduced in the T3, T2, and T1 groups at 3.8 ± 0.41, 2.8 ± 0.41, and 2.3 ± 0.52, respectively. At 3 dpi, the lesion score was statistically significant between T4 and the other infected groups. At 5 dpi, the lesion score was significantly higher in the T3 and T4 groups compared with those in the T1 and T2 groups. At 10 dpi, the lesion score was significantly higher in the T4 group compared with that in the T1 group (Figure 2).

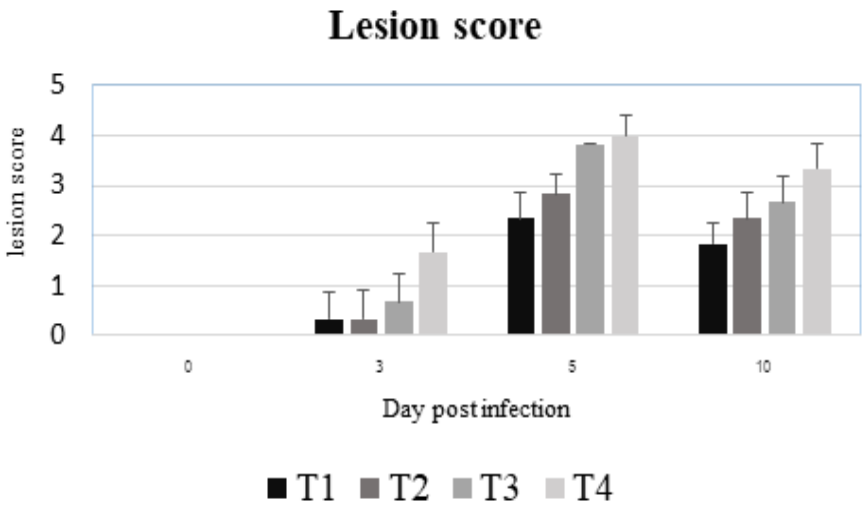

Figure 2 Lesion score of broiler chickens infected with different dosages of *Eimeria tenella*. Lesion score was measured in the T1 (treatment 1; challenged with 100 sporulated oocysts of *E. tenella*); T2 (treatment 2; challenged with 1,000 sporulated oocysts of *E. tenella*); T3 (treatment 3; challenged with 10,000 sporulated oocysts of *E. tenella*); T4 (treatment 4; challenged with 100,000 sporulated oocysts of *E. tenella*). Different letters at the same time point represent significantly different ($P<0.05$) by unpaired t-test.

Observations on the endogenous phage

There was no *E. tenella* at any stage in the specimens of all groups at 0 dpi. The parasite burden levels in the H&E sections at all stages were proportional to the infectious doses, which were highest in the T4 group and lowest in the T1 group. The time point to detect the endogenous stages was similar among the infected groups. At 3 dpi, schizonts that contain asexual stages of *E. tenella* were found in the specimens of all infected groups (Figure 3B, F, J, and N; Table 1). At 5 dpi, both asexual and sexual stages (schizonts, microgametocytes, and macrogametocytes) were detected in the specimens of all infected groups (Figure 3C, G, K, and O; Table 1). At 10 dpi, only oocysts were found in the specimens of all infected groups (Figure 3D, H, L, and P).

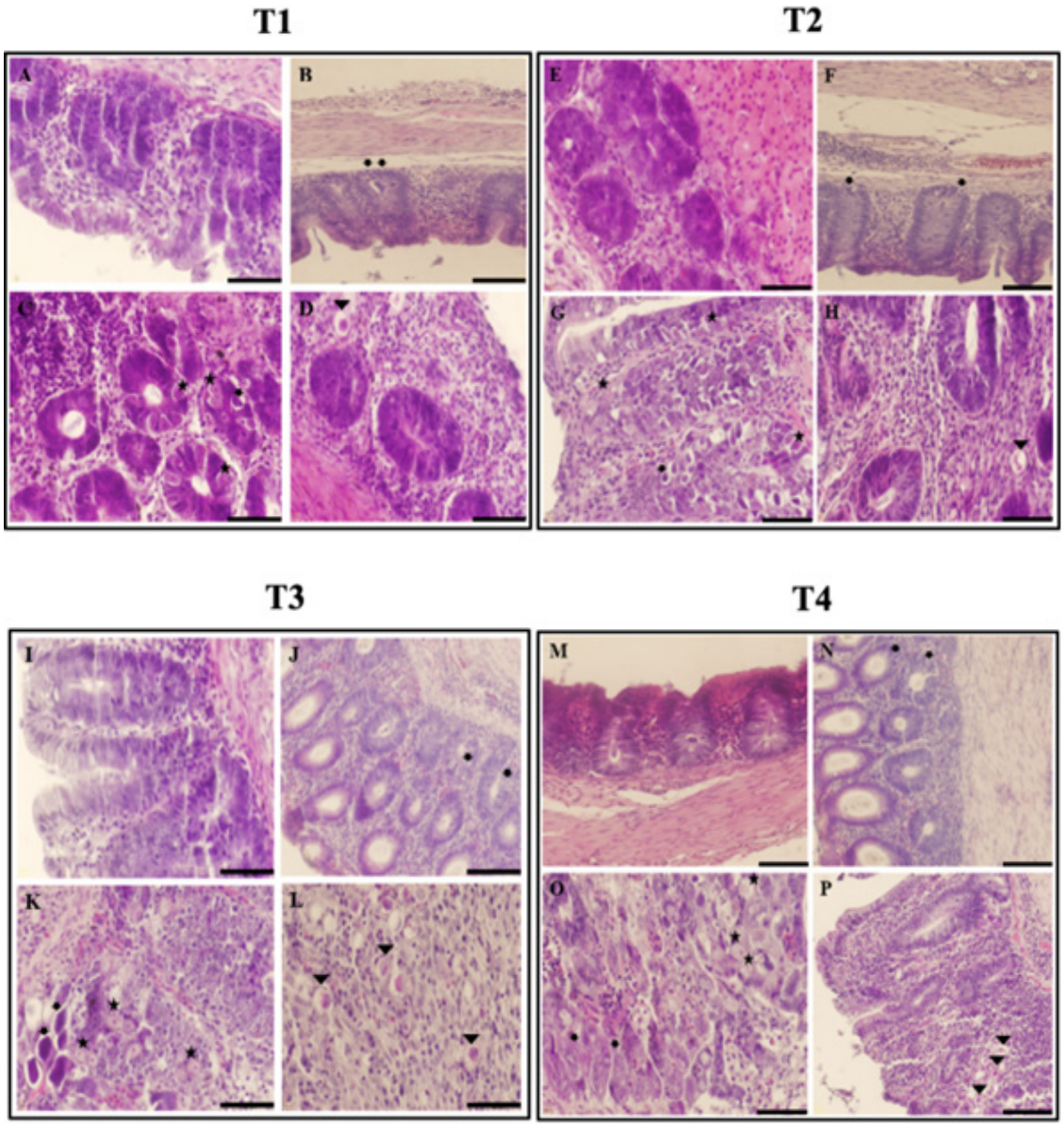

Figure 1 The observations of the endogenous phage shown by HE stains. HE-stained specimens were observed under light microscopy. The arrowheads indicate the immature oocyst. The arrows indicate the schizonts. Stars indicate gametogony stage. Bar scale is 50 µm. T1 challenged with 100 sporulated oocysts of *E. tenella* (A, B, C, D was sectioned at 0, 3, 5, 10 dpi, respectively); T2 challenged with 1,000 sporulated oocysts of *E. tenella* (E, F, G, H was sectioned at 0, 3, 5, 10 dpi, respectively); T3 challenged with 10,000 sporulated oocysts of *E. tenella* (I, J, K, L was sectioned at 0, 3, 5, 10 dpi, respectively); T4 challenged with 100,000 sporulated oocysts of *E. tenella* (M, N, O, P was sectioned at 0, 3, 5, 10 dpi, respectively).

Table 1 The developmental stages of different dosages *E. tenella* in the cecum of infected chickens

Time (dpi)	Asexual stages				Sexual stages			
	T1	T2	T3	T4	T1	T2	T3	T4
0	0	0	0	0	0	0	0	0
3	++	++	+++	+++	0	0	0	0
5	+	++	++	++	+	++	+++	+++
10	0	0	0	0	+	+	+	+

Note: The developmental stages were measured using H&E staining sections. The results were calculated as: +, 1 to 10 cluster in the infected cecum section; ++, 10 to 30; +++, >30. Histological slides were evaluated by two individual researchers with technical skills in histopathology under blinded conditions.
T1 (treatment 1; challenged with 100 sporulated oocysts of E. tenella);
T2 (treatment 2; challenged with 1,000 sporulated oocysts of E. tenella);
T3 (treatment 3; challenged with 10,000 sporulated oocysts of E. tenella);
T4 (treatment 4; challenged with 100,000 sporulated oocysts of E. tenella

DISCUSSION

The chickens in the T4 group excreted oocysts in the feces one day earlier than the other groups. The time when chickens begin to shed oocysts in feces after infection depends on many factors, such as immunity, age of chickens, and *Eimeria* spp. (Shirley et al., 2005). There have been many studies on the crowding effect on oocyst pattern and lesion score (Lillehoj, 1988; Williams, 2001; Soutter et al., 2021); however, to our knowledge, this report is the first to address the result of higher infectious doses in early oocyst excretion in the feces of infected chickens. The results of this study are not consistent with the study of Choi et al. (2021), which examined the effects of 4 different oocyst doses of *E. tenella* (6,250; 12,500; 25,000, and 50,000 sporulated oocysts of *E. tenella)*. The author reported that oocysts were detected in the feces of four groups at 5–8 dpi. This difference may be due to the fact that our study used a higher infectious dose (100,000 sporulated oocysts of *E. tenella* compared with 50,000 oocysts in Choi et al.'s study).

The life cycle of *E. tenella* involves an exogenous phase that occurs in the environment and an endogenous phase that takes place within the chicken's cecum. During the exogenous phase, oocysts excreted by infected chickens are released into the environment. These oocysts undergo a process called sporulation, where they differentiate, and become infective. In the endogenous phase, the parasite undergoes several stages of reproduction and differentiation. First, there are three rounds of asexual reproduction, known as schizogony. During schizogony, the parasite multiplies extensively, resulting in an increased number of infected cells. Following schizogony, the parasite undergoes sexual differentiation, known as gametogony. Male and female gametes are formed, which subsequently fertilize, leading to the formation of zygotes. These zygotes develop into unsporulated oocysts. Finally, the unsporulated oocysts are shed from the infected chicken's cecum into the environment, where they undergo sporulation, and become infective, starting the exogenous phase of the life cycle once again (Shirley et al., 2005). Thus, oocysts were detected in the

feces of chickens in the T4 group earlier than in the other groups, possibly due to the effect on asexual reproduction, sexual differentiation, or the process of eliminating the oocyst from the cell.

We examined the lesion score in the cecum and the developmental stages of *E. tenella* in the tissue by H&E staining on days 0, 3, 5, and 10 after the oocyst challenge to determine at which stage the infectious dosages affect the growth of the parasite. The results of the lesions score examination showed that in all infected groups, lesions began to appear at 3 dpi, were most severe at 5 dpi, and recovered at 10 dpi. Besides, the time point to detect the intracellular reproductive stages was similar among the infected groups. At 3 dpi, schizonts containing asexual stages of *E. tenella* were found in the specimens of all infected groups (Figure 3). At 5 dpi, both asexual and sexual stages (schizonts, microgametocytes, and macrogametocytes) were detected in the specimens of all infected groups (Figure 3). Thus, it can be seen that the development of the endogenous phase in the chicken cecum between infected groups is similar. Our results are consistent with the work of Soutter et al. (2021), who reported that no significant differences were found in the lesion score and parasite replication between three chicken lines after challenging three doses (250; 4,000; 8,000, and 12,000 of sporulated oocyst). Remarkably, microscopic examination showed that the gametogony stage was found at 5 dpi in all infected groups; however, only the T4 group showed oocysts in the feces at 5 dpi. Therefore, the infectious dosages may not affect the sexual and asexual reproduction stages but affect oocyst release from the intestinal epithelium. Further study is needed to clarify the mechanisms by which high infectious dosages promote oocyst shedding in the feces.

CONCLUSIONS

Overall, chickens infected with high oocyst dosages experienced an acceleration of the excretion of oocysts in the feces. Oocyst dosage does not affect the asexual and sexual reproductions of *E. tenella* in intestinal epithelial cells but may influence the excretion of oocysts from the intestinal tissue.

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AUTHOR CONTRIBUTIONS

Ho Thi Dung, Pham Hoang Son Hung; Conceptualization and design the experiment, investigation, supervision, editing, and finalization
Ho Thi Dung, Pham Hoang Son Hung, Nguyen Thi Thuy, Le Dinh Phung, Nguyen Thi Hoa; Investigation, methodology, formal analysis, and manuscript preparation

CONFLICT OF INTEREST

We have no conflict of interest.

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