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RESEARCH ARTICLE
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The comparative analysis of infection pattern and oocyst output in *Eimeria tenella, E. maxima and E. acervulina* in young broiler chicken

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Abstract

Aim: To assess the pattern of infection pattern by artificially infected *Eimeria* strains of Korean isolates and assessed the degree of sporulating oocysts at different temperature.

Materials and Methods: Birds were orally inoculated with oocysts of *Eimeria tenella*, *Eimeria maxima* and *Eimeri acervulina.* Oocyst count, oocyst isolation and sporulation were evaluated from the fourth to the tenth day post infection. Histopathological studies also made in the caecum and intestinal to comparative lesions.

Results: Mean oocyst counts of these species increased more quickly on the day of 5th to 6th of post-infection. *E. acervulina* reached its highest infection level on the 6th day while both *E. tenella* and *E. maxima* had peak on the 7th day. The prepatent period of oocyst output in the *E. acervulina* started 120 h and ended on the 10th day after inoculation, whereas the *E. maxima* started oocyst output at 144 h and *E. tenella* oocyst output at 168 h respectively. The best temperature for optimum sporulation was found to be at 25^{0}C at sporulation rate of 88.91%, while at 20^{0}C sporulated 88.03%, and at 30^{0}C sporulated 82.44%.

Conclusion: Current study represent the pattern of infection, pathogenesis and optimum sporulation temperature and our results suggested that 25^{0}C is optimum for sporulated oocyst of Korean isolates of *E. tenella*, *E. maxima* and *E. acervulina.*

Keywords: broiler chick, *Eimeria,* oocyst, pathogen.

Introduction

Coccidiosis is a universally important disease of poultry production. Chicken flocks free from coccidia are extremely rare and at least three species of *Eimeria* (*E. tenella, E. maxima* and *E. acervulina*) are commonly found in all commercial chickens [1]. The protozoan parasites of the genus *Eimeria* multiply in the intestinal tract and cause tissue damage, resulting in interruption of feeding and digestive processes or nutrient absorption; dehydration; blood loss; and increased susceptibility to other disease agents [2]. In India, estimation has revealed that commercial broiler industry is a major suffererdue to coccidiosis wherein 95.61% of the total economic loss occurs due to the disease [3]. The seven species (*E. acervulina*, *E. brunetti*, *E. maxima*, *E. mitis*, *E. necatrix*, *E. praecox*, *and E. tenella*) have different properties regarding localisation in the gut, pathogenicity, pre-patent period, fecundity and immunogenicity. Diagnosis of coccidiosis is based on comparison of clinical features, gut pathology in the host, and parasite properties such as morphology of different parasite stages in feacal material or intestine and pre-patent period [4].

The development of sexual stages (micro-and macro-gametocytes) and formation of oocysts were observed within epithelial cells of the small intestine (duodenum, jejunum) after infection with *E. maxima* (144 h post infection (PI) and *E. acervulina* (96 h PI) and of the caecum after infection with *E. tenella* (136 h PI) [5].This intracellular parasite has a complex life-cycle, where it passes through asexual and sexual stages of development. The final stage of development, the oocyst, is excreted from the host and, under the appropriate conditions of temperature and humidity, undergoes a process known as sporulation, where it becomes infective.

The infective form of *Eimeria* is the highly resistant oocyst, which is shed in the feces of infected animals. *Eimeria* species identification is based on clinical features, morphological and biological features as sizes of oocysts, sites of infection, pre-patent period, sporulation time. *E. maxima* can be easily identified based on oocyst size, while *E. tenella* and *E. necatrix* produce unmistakable lesions [6].

The present experiment was undertaken to study the pattern of infection and oocyst output of *Eimeria* strains which were isolated in artificially infected situation in Korea, and assessed the degree of sporulation oocysts at different temperature. We also examined the histo-pathological observations in infection of each *Eimeria* strain.

Materials and Methods

Ethical approval: Experiment was carried out in

accordance with the guidelines approved by the Chonbuk Animal Care and Use (Approval no. CBU 2011-0064).

Parasites and birds: Oocysts of *E. tenella (*Fj447468*)*, *E. acervulina* (FJ447467) and pure strain *E. maxima* were obtained from the Laboratory of Vererinary Parasitology, College of Veterinary Medicine, Chonbuk National University (Jeonju, Republic of Korea). These strains were propagated in chicks; oocysts were preserved in 2.5% potassium dichromate solution to induce sporulation at 28^{0}C in water bath for 4-7 days. Then three of these isolates, *E. tenella (*FJ447468, South Korea*)*, *E. acervulina* (FJ447467, South Korea) and pure strain *E. maxima* had been stored in 2.5% potassium dichromate at 4^{0}C until use.Fifty-one day-old broiler chicks (Samhwa breeding co.; Ross) were fed with coccidian-free food. On 14 days, 45 healthy chicks were chosen for the following experiments.

Experimental design: Sporulated oocysts were isolated in the 2.5% potassium dichromate, and washed three times with distilled water, and counted using a MacMaster method under a light microscope. Artificial infestation was done as describe by You [7]. Shortly, each of 14 days old chicks was divided into 3 groups of 15 individuals and was infected orally with a suspension of 1.0x10^4 oocysts/chick from a stock of *E. tenella* (chickens group I), *E. maxima* (chickens group II) and *E. acervulina* (chickens group III) per bird, respectively. The chickens were given unlimited access to food and water and constant light was provided for the duration of the experiment. Fecal materials were collected from 5 to 10 days post-infection, and the number of oocysts was assessed using a MacMaster method counting chamber. Total oocyst numbers were calculated and histopathological examinations were evaluated. Pathological lesion, oocyst isolation and sporulation was done as describe by Amer *et al.*[8].

Oocyst count: Oocyst count per gram of fecal material (OPG) was evaluated from the fourth to the tenth day PI. All the feces of each chicken group were collected daily and OPGs were counted using the MacMaster method counting technique [9].

Oocyst isolation and sporulation: After 120 h of the post infection, fecal samples were collected and isolated oocysts following our laboratory's procedures. Briefly, chicken fecal was mixed in the water (1:2 w/v) and filtered by mesh (~1 mm). Suspension of feces was centrifuged at 3000 rpm for 10 min in a 50-ml centrifuge tube, the supernatant was removed, and the pellet was resuspended with sucrose solution (128g granulated sugar + 100ml tap water) and on vortex and centrifuged at 3000 rpm 10 min. Supernatant was collected, and was added with 10 times water and centrifuged at 3000 rpm 10 min. After removing the supernatant, the pellet was transferred into a 50-ml centrifuge tube and centrifuged at 3000 rpm 10 min. Pellet was resuspended with a similar volume of saturated salt solution (~400g Salt + 1000ml DW; 1.18-1.2 species gravity), and on vortex and centrifuged at 3000 rpm 10 min. Supernatant were then transferred into new 50 ml centrifuge tubes, 10 times water was added and centrifuged at 3000 rpm 10 min. Finally pellets were resuspended in 2.5% $K_2Cr_2O_7$.

Pure oocysts (*E. tenella*, *E. maxima* and *E. acervulina*) were incubated at 20^{0}C, 25^{0}C, 30^{0}C and 35 ^{0}C for 6 days, then the sporulation rates were determined by counting 100 oocysts under x100 magnification (four replications) at 24, 48, 72, 96, 120 and 144 h of sporulation, respectively. Sporulation was considered to be completed when sporozoites within the sporocysts were identified and the Stieda body, which appeared as a clear protuberance at the narrow end of the sporocysts, was visible. The number of sporulated oocysts was calculated by the number of harvested oocysts and their sporulation rate.

Histopathological examination: Histopathological studies were made in the caecum and intestinal (jejunum, duodenum) two randomly chosen chicks per group were killed every day between 3rd and 6th days PI. Tissues were taken from the mid-jejunum, duodenum and caecum and fixed in 10% formalin solution. After fixation, samples were dehydrated in alcohol, cleared in xylene, finally the specimens were embedded in paraffin wax, sectioned at 5 μm and stained by haematoxylin and eosin (H&E), mounted and examined under a light microscope.

Results

Sporulation and physical condition of oocysts: To provide quantitative data, the structural integrity of oocyst walls, sporocysts and sporozoites were assessed microscopically. Figure-1 comprises photomicrographs of sporulated and unsporulated oocysts of Eimeria species. The double oocyst-walls were undamaged and internal structures were well enough preserved and the sporocysts and sporozoites can be seen in detailed description. Sporulation rates of oocysts obtained from different condition temperatures were given in Figure-2. *Eimeria* oocysts sporulated most efficiently under the different temperature conditions studied, and that the lowest sporulation was observed in the samples with the highest temperature condition (Figure-2). On 6th day, the mean sporulation rate of 35^{0}C was significantly lower than that of 20^{0}C, 25^{0}C and 30^{0}C. The best temperature for complete sporulation was found to be 25^{0}C, sporulation rate was 88.91%, while 20^{0}C sporulated 88.03%, and 30^{0}C sporulated 82.44%.

Oocyst count: More irregular courses of oocyst excretion were observed with *E. tenella*, *E. maxima* and *E. acervulima* (Figure-3). Mean oocyst counts of these species increased more quickly on the day of 5th to 6th PI. *E. acervulina* reached its highest infection level on the 6th day while both *E. tenella* and *E. maxima* reached a maximum infection peak on the 7th day. The prepatent period of oocyst output in the *E. acervulina*

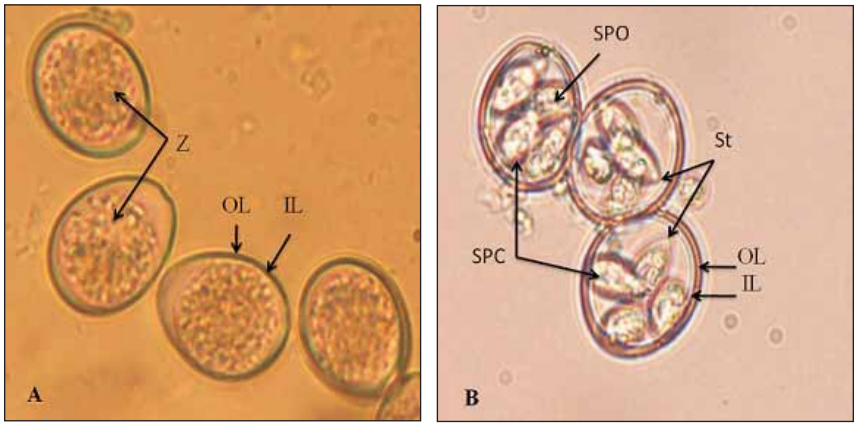

Figure-1: Oocysts morphological feature: **(A)** Photomicrograph of freshly shed unsporulated oocyst (Z-zygote); in the ovoidal was shown. Two layers of the oocyst wall (OL-outward, IL-inward). x 400.; **(B)** Photomicrograph of the sporulated spherical oocyst with four sporocysts (SPC) each containing two sporozoites (SPO); the stieda body (St). x400.

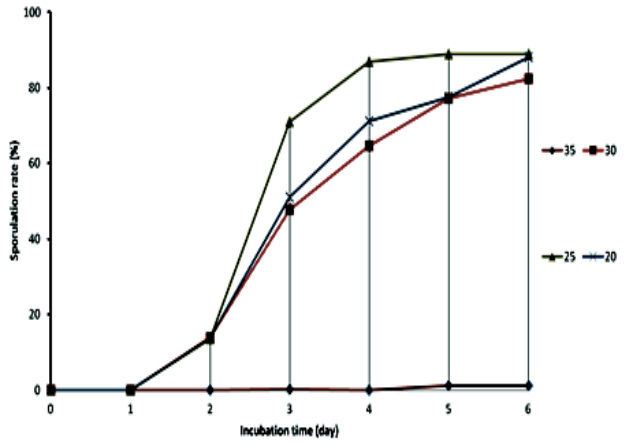

Figure-2: Mean sporulation rate of *Eimeria* in water bath with different temperature.

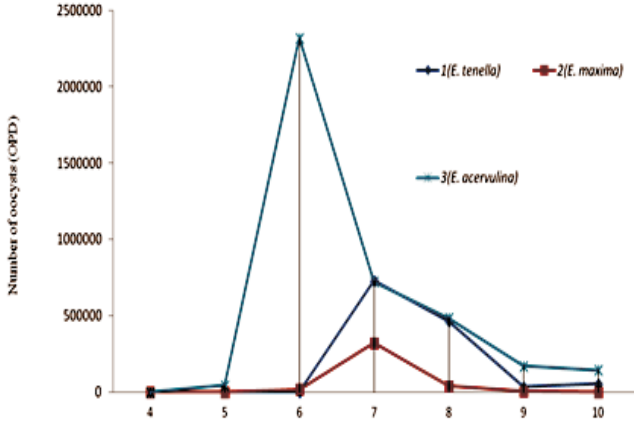

Figure-3: Fecal oocysts shedding in chickens infected with *Eimeria tenella, E. acervulina* and *E. maxima*. Each data point shows the average oocysts of four counts.

started 120 h after oocyst inoculation and ended on the 10th day after inoculation, whereas the *E. maxima* started oocyst output at 144 h and *E. tenella* oocyst output at 168 h, respectively. On the other hand, chickens from infected group 3 showed higher *E. acervulina* oocyst shedding (2.32 x 10^6/g faeces) than infected *E. tenella* from group 1 (0.7 x 10^6/g faeces) and group 2 (*E. maxima)* (0.3 x 10^6/g faeces), (Figure-3). After it reached its peak, the number of oocysts gradually reduced and a small number of remained on the 8th day PI.

Histopathological observations of chickens infected with the Eimeria tenella, *E. acervulina* and *E. maxima* during the course of infection: Histopathological examination of tissue sections at the 72, 96, 120 and 144 h PI (Figure-4, 5, and 6) revealed the detection of developmental *Eimerial* stages in duodenum, jejunum and cecum, respectively. In the chicken inoculated with *E. tenell*a, gametocytes were observed within crypt epithelial cells of the cecum at 72 h PI (Figure-4.B), then were observed numerous intracellular schizonts containing merozoites and immature macrogametocytes in the border epithelial cells of cecum at 120 and 144 h PI (Figure 4-C and D). So, after 72 and 120 h of *E. tenella* infection in chickens, severe inflammatory process was observed in the lamina propria and among crypts. From 96 h PI, increasing number of second mature schizonts of *E. maxima* was present within border cells of villi of the jejunum. Fourth generation schizonts, oocysts, zygotes and gametocytes were seen from 144 h PI present within the submucosa cells of the mid-jejunum (Figure-5). Sections from the duodenum at 72 and 96 h PI, increased numbers of second schizonts, young macrogametocyte and immature gametocytes in the intestine cells (Figure-6). Infected *E. acervulina* that at 120 h, great multiplication of fourth-generation schizonts and sexual stages in the middle and near the lumen of the crypts were also observed with hyperplasia of the villi. The schizont was observed 1-2 days before the maximum oocyst output.

Discussion

In all experiments, there was a successful infection with *Eimeria* species as indicated by the intestinal lesions and the shedding of the oocysts. The experimental infection of chickens by *Eimeria* species isolates is aimed to study the biological characters of each isolate and confirming the diagnosis of each species of Eimeria .The protocol was also followed by Amer [8] and Kucera [10]. The histopatholological lesions, mean OPG of the infected groups of chickens at day 7th PI were consistent with findings of Jordan and

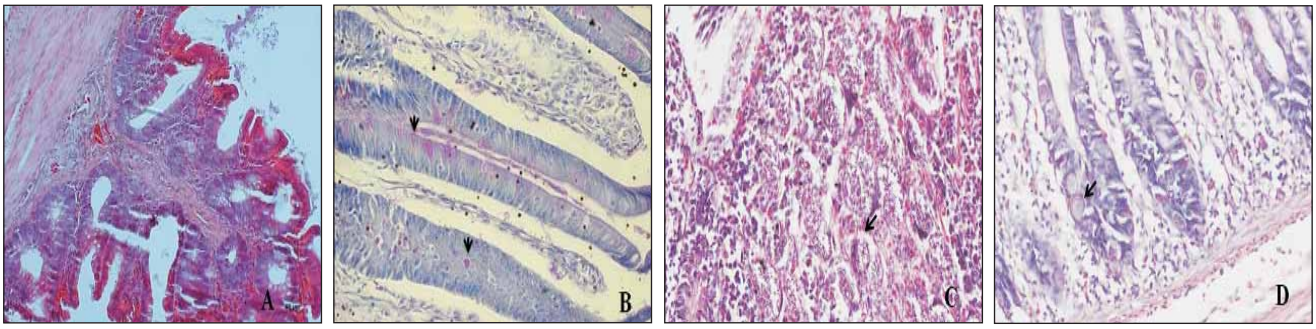

Figure-4: Comparative histopathological observations of chicken intestine post infection (PI) with *E. tenella* ($1.0x10^4$ sporulated oocyst/chick): oocyst's developmental stages in the cecum cells (AXIO ZEISS Imager M2 microscope, using AxioCam HRc). (A) Photomicrograph of a cecum from broiler chicken of the control. Cecum of non infected: normal tissues can be seen (H & E, bar=20 µm) x200; (B) Photomicrograph of a cecum from a broiler chicken inoculated with *E. tenell*a at 72 h PI: gametocytes in the cecum cells (H&E) x200; (C) Photomicrograph of a cecum from a broiler chicken inoculated with *E. tenell*a at 120 h PI: Numerous intracellular schizonts containing merozoites (arrow) can be seen with severe submucosal hemorrhage. (H&E). x400; (D) Photomicrograph of a cecum from a broiler chicken inoculated with *E. tenell*a at 144 h PI: infected cells with immature macrogametocytes in the border epithelial cells of cecum. (H&E). x400. Arrow indicates different forms of the intracellular parasite.

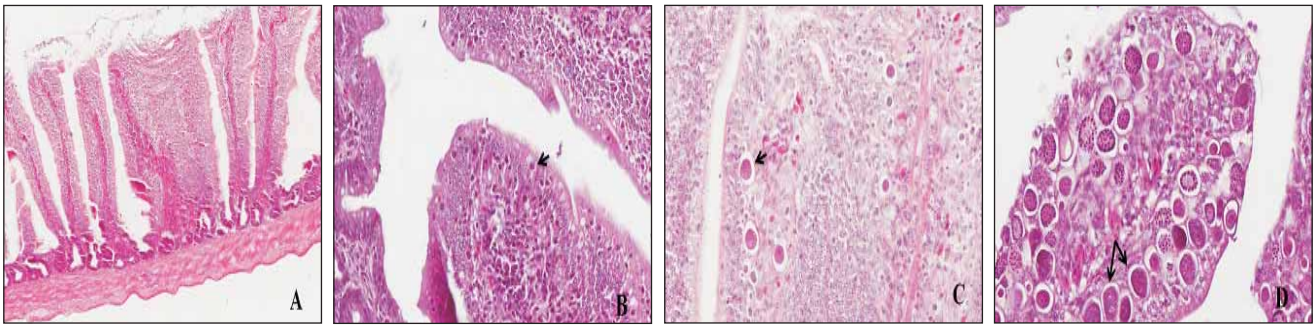

Figure-5: Jejunum sections of chicken infected with oocysts *E. maxima* ($1.0x10^4$ sporulated oocyst/chick) showing: oocyst's developmental stages in the jejunum cells. (A) Photomicrograph of a jejunum from broiler chicken of the control. Normal jejunum can be seen. (H&E) x80; (B) Photomicrograph of a jejunum from a broiler chicken inoculated with *E. maxima* at 96 h PI: presence of second mature schizonts in the border cells of villi. (H&E) x400; (C) Photomicrograph of a jejunum from a broiler chicken inoculated with *E. maxima* at 120 h PI: zygotes and oocysts in the cells. (H&E) x400; (D) Photomicrograph of a jejunum from a broiler chicken inoculated with *E. maxima* at 144 h PI: fourth generation schizonts, oocysts, zygotes, gametocytes in submucosa cells. (H&E). x400. Arrow indicates different forms of the intracellular parasite.

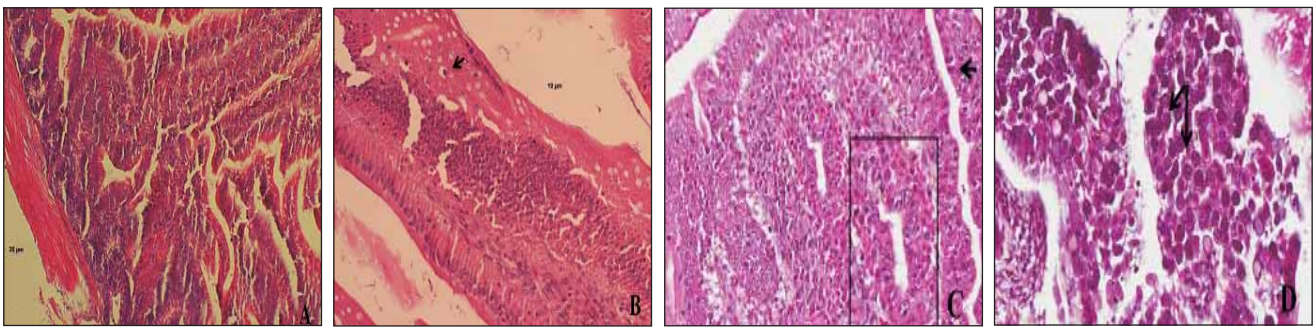

Figure-6: Chicken duodenal sections stained with H&E of birds infected with oocysts *E. acervulina* ($1.0x10^4$ sporulated oocyst/chick) showing: oocyst's developmental stages in the duodenum cells. (A) Photomicrograph of a duodenum from broiler chicken of the control. Normal duodenum can be seen. (H&E) x200; (B) Photomicrograph of a duodenum from a broiler chicken inoculated with *E. acervulina* at 72 h PI: presence of second schizonts and young macrogametocyte in the striated border cells of villi. (H&E, bar=10 µm), x400; (C) Photomicrograph of a duodenum from a broiler chicken inoculated with *E. acervulina* at 96 h PI: presence of immature gametocytes in intestine cells. (H&E) x400; (D) Photomicrograph of a duodenum from a broiler chicken inoculated with *E. acervulina* at 120 h PI: great multiplication of micro and macrogametocytes, oocysts, zygotes, fourth generation schizonts in the middle and beginning of crypts and hyperplasia up to the tip level of villi. (H&E), x400. (E) Photomicrograph of a duodenum inoculated with *E. acervulina* at 120 h PI: x400 extension (x4). Arrow indicates different forms of the intracellular parasite.

Pattison in chickens infected with coccidian [11]. Prepatent periods may generally range from 4 to 5 days post infection. Maximum oocyst output ranges from 6 to 9 days PI.

Edgar [12] reported that $29^0C\pm1^0C$ (84^0F) was optimum for most rapid sporulation of *E. tenella* and that some oocysts reached the infective stage between 15 and 18 h. In the present study, at the first observations the more high temperature samples had a much lower sporulation rate, and Figure-2 indicate that sporulation started later in high temperature conditions than in low temperature conditions.

The use of 2-3% potassium dichromate for sporulation at room temperature has been documented in many reports. Under this condition, at least 95% of the oocysts sporulate in 7-10 days. In our study, the best temperature for complete sporulation was found to be 25^0C in 6 days. The sporulation time may decrease in crowds of oocysts or depend on contamination. This may support the opinion that different sporulation times may be related to different experimental factors or laboratory techniques or to the lack of adequate oxygen. Once sporulation started, there were no differences in the rate of sporulation up to the maximum sporulation proportion obtained at different moisture contents. Tierney *et al.* [13] reported that the development of *E. tenella* continued to immature and mature schizonts in MDBK cells. These stages were observed in the present study at 48 and 60 h respectively. This sporulation time also support the study of Al-Quraishy *et al* [14]. But in our histopathological study, observed at 120 h PI in broiler chickens.

Researchers used different criteria to evaluate coccidial infections. Some suggested that oocyst production might be a very unreliable quantitative criterion [15] as the number of oocysts produced is affected by factors such as the inherent potential of each species to reproduce in a non-immune host; immunity or resistance developed by the host; the 'crowding' factor; competition with other species of coccidia or other infectious agents; nutrition of the host; and strain differences of the host. The inherent difference in reproductive potential is high for *E. tenella*, and *E. acervulina*, and low for *E. maxima*. Immunity, which is specific to each coccidian species, results in decreased production of oocysts after ingestion of infective oocysts [16].

The histopathological analyses confirmed more extensive presence of lesions, observed with the light microscope, where more inflammatory cells occur in chickens infected with *E. tenella* than in *E. maxima*, suggesting a low pathogenicity of *E. acervulina*. This criterion was used previously by Karim *et al.* [17] who identified 5 Eimeria species; *E. acervulina, E.tenella, E. maxima, E. brunette and E. necatrix*; based on a lesion seen at post mortem examinations of naturally infected birds, dimensions of oocyst and lesion seen in experimentally infected chicks with single oocyst. The histological finding in this study confirmed the diagnosis of each species as *E. acervulina* showed presence of gametocyte with the characteristic inflammatory cells in duodenal part of intestine. The fact which is agreed with Hein [18] and Asaduzzaman *et al* [19]. *E. tenella* showed considerable numbers of oocyst in lamina propria of coecum beside sever hemorrhage and complete desquamation of epithelium and edema of muscular tissue which agreed with the finding of Levine [20].

The entire lamina propria revealed severe haemorrhages, necrosis and disintegration of glandular epithelial cells. Several schizonts were observed in the epithelial cells along with merozoites, infiltrating neutrophils and eosinophils.

Conclusion

In conclusion, infection through gavage with a single high dose of inoculum has no similarity with the field situation. Infection through the litter mimics the field situation in combination with controlled conditions and allowing experimental flexibility and a large number of experimental units within the research facility.

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Competing interests

The author declare that they have no competing interests.

References

1. Williams, R. B. (2002) Anticoccidial vaccines for broiler chickens: pathways to success. *Avian Pathol.,* 31(4): 317-353.
2. Morris, G. M. and Gasser, R. B. (2006) Biotechnological advances in the diagnosis of avian coccidiosis and the analysis of genetic variation in *Eimeria*. *Biotechnol Adv,* 24(6): 590-603.
3. Beraa, A.K., Bhattacharyaa D., Pana, D., Dharab, A., Kumarc, S.and Dasa, S.K. (2010) Evaluation of economic losses due to doccidiosis in poultry industry in India. *Agri Econ Res Rev,*23 (1): 91-96.
4. Haug, A., Thebo, P. and Mattsson, J. G. (2007) A simplified protocol for molecular identification of *Eimeria* species in field samples. *Vet Parasitol,* 146(1-2): 35-45.
5. Belli, S. I., Ferguson, D. J., Katrib, M., Slapetova, I., Mai, K., Slapeta, J. and Smith, N. C. (2009) Conservation of proteins involved in oocyst wall formation in Eimeria maxima, Eimeria tenella and Eimeria acervulina. *Int J Parasitol,* 39(10): 1063-1070.
6. Hadipour, M.M., Olyaie, A., Naderi, M., Azad, F., Nekouie, O. (2011) Prevalence of *Eimeria* species in scavenging native chickens of Shiraz Iran. *Afr J Microbiol Res*, 5(20): 3296–3299.
7. You, M. (2014) Detection of four important *Eimeria* species by multiplex PCR in a single assay. *Par Int*, 63 (3), 527-532.
8. Amer, M.M., Awaad, M.H.H., Sherein-Said, A., Ghetas, M. M. and Kutkat, M.A.(2010) Isolation and identification of eimeria from field coccidiosis in chickens.*J Ame Sci*, 6 (10): 1107-1114.
9. Long, P. L., Joyner, L. P. Millard, B. J. and Norton, C. C. (1976) A guide to laboratory techniques used in the study and diagnosis of avian coccidiosis. *Folia Vet Latina*, 6(3): 201-217.
10. Kucera, J. (1990) Identification of Eimeria species in Czechoslovakia. *Avian Pathol,* 19(1): 59-66.
11. Jordan, F. T. W. and Pattison, M. (2008) Protozoal infection. In: McDougald, L.R., editor. Polutry Diseases. 8th edition, Philadelphia, P.A. p974-978.
12. Edgar, S. A. (1955) Sporulation of oocysts at specific temperatures and notes on the prepatent period of several species of avian coccidia. *J Parasitol*, 41(2): 214-216.
13. Tierney, J. and Mulcahy, G. (2003) Comparative development of *Eimeria tenella* (*Apicomplexa)* in host cells in vitro. *Parasitol Res*, 90(4): 301-304.
14. Al-Quraishya, S., Abdel-Bakia, A.S and Dkhila, M.A. (2009) Eimeria tenella infection among broiler chicks Gallus domesticus in Riyadh city, Saudi Arabia. *J King Saud Univ Sci,* 21(3):191–193.
15. Oikawa, H., Kawaguchi, H., Nakamoto, K. and Tsunoda, K.

(1975) Field surveys on coccidial infection in broilers in Japan-results obtained in autumn and winter and summarized in 1973. *Nihon Juigaku Zasshi,* 37(3): 271-279.
16. Arabkhazaeli, F.,Nabian,S., Modirsaneii, M.,Mansoori,V and Rahbari,S. (2011) Biopathologic characterization of three mixed poultry *Eimeria* spp. Isolates. *Iran J Parasitol*, 6 (4): 23–32.
17. Karim, M.J. and Trees, A.J. (1990) Isolation of five species of Eimeria from chicken in Bangladesh. *Trop Ani Health Prod,* 22 (2):153-159.
18. Hein, H. (1971) Pathogenic effects of *Eimeria necatrix* in young chickens. *Exp Parasitol,* 30(3): 321-330.
19. Asaduzzaman, M., Miah, M. S., Siddika, A., Popy, N. and Hossain, M. M. (2011) Experimental production of necrotic enteritis in broiler chickens. *Bangl J Vet Med,* 9(1): 33–41.
20. Levine, N.D. (1985) *Erhardorina n. g, Ascogregarina polynesiensis n.* spp.*, Eimeria golemanskii n.* spp.*, Isospora tamariscinin.* spp.*, Gregarina kazumii n. nom*., new combinations and emendations in the names of apicomplexan protozoa. *J Protozool,* 32(3):359-63.

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