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An animal model using *Eimeria* live vaccine and to study coccidiosis protozoa pathogenesis

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Abstract : Cell culture systems for the protozoan *Eimeria* are not yet available. The present study was conducted to develop an animal model system by inoculating animals with a live *Eimeria* vaccine. This study was conducted on 3-day-old chickens (n = 20) pretreated with cyclophosphamide. The chickens were divided into 2 groups: the control group (n = 10) and the inoculated group that received the live *Eimeria* vaccine (n = 10). During the study period, we compared the clinical signs, changes in body weight, and number of oocysts shed in the feces of the control and inoculated group. This study showed that oocyst shedding was significantly higher in the chickens inoculated with live *Eimeria* oocysts than in the control chickens. Moreover, body weight gain was lesser in the animals in the inoculated group than in the control animals. Fecal oocyst shedding was observed in the inoculated animals. On the basis of these findings, we suggest that live *Eimeria* vaccination with cyclophosphamide pretreatment may be used to obtain an effective animal model for studying protozoan infections. This animal study model may eliminate the need for a tedious continuous animal inoculation process every 6 months because the live coccidiosis vaccine contains live oocysts.

Keywords : animal model, coccidiosis, cyclophosphamide, *Eimeria*, Protozoa

Eimeria infection causes extensive destruction of the intestinal epithelium, resulting in a reduction in feeding efficiency and body weight gain and a temporary reduction in egg production [5, 10]. Although coccidiosis can be mainly controlled by using chemotherapeutic agents, novel treatment approaches are urgently needed because of the increasing emergence of drug-resistant parasitic strains in commercial poultry production settings [1, 2, 17]. The genus *Eimeria* includes obligate intracellular parasites, which are challenging to study. *In vitro* culture has become a useful study tool because it enables us to understand the aspects of the organisms' life cycle that could not otherwise be studied [9]. Thus far, no efficient *in vitro* model is available for propagating all the life cycle stages of the protozoan *Eimeria*. For the efficient storage of sporulated *Eimeria* oocysts they must be maintained by infecting chickens every 6 months as described; however, this maintenance is tedious and time-consuming [16]. A cell culture system for *Eimeria* species is not yet available. The present study was conducted to develop an animal model system by inoculating animals with a live *Eimeria* vaccine.

This study was conducted on 3-day-old chickens (n = 20) at the animal facility of the Center for Animal Resources Development, Wonkwang University, Korea. The animals were acclimatized and kept in an animal facility with regulated temperature (28 ± 2°C), humidity (50 ± 5%), and light/dark cycle (12/12 h). The animals were fed commercial post-broiler diet without antibiotics and coccidiostats (Hanil Feed, Korea) and tap water *ad libitum*. The chickens were kept in wire-floored grower cages during the study period. All animals were pretreated with cyclophosphamide (Sigma Chemical, USA). The cyclophosphamide (cyclophosphamide 2H-1.3, 2-oxaza-phosphorin-2-amine)-pretreated chickens were injected a solution containing 3 mg/mL cyclophosphamide subcutaneously in the abdominal region daily for 4 consecutive days. Thereafter, the chickens were divided into 2

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groups: the control group (n = 10) and the inoculated group that received the live *Eimeria* vaccine (n = 10). Chickens in the inoculated group were orally inoculated with the live *Eimeria* vaccine, Coccivac-D (Intervet/Schering-Plough Animal Health, USA), which contains 16,000 live oocysts of *Eimeria* (*E.*) *acervulina, E. mivati, E. maxima, E. tenella, E. necatrix, E. praecox, E. brunette,* and *E. hagani*. Chickens in the inoculated group (n = 10) were orally inoculated by gavage by using a 24-gauge stainless-steel animal-feeding tube (Popper & Sons, USA), which is used for feeding mice, attached to a 3 mL syringe (Fig. 1). The oral infectious dose of this vaccine has been found to be approximately 10^4 oocysts of *Eimeria* species in 1 mL of saline. The control chickens (n = 10) received saline through the same route.

During the study period, the animals were checked twice daily for morbidity and mortality. Further, we compared the clinical signs, changes in body weight, number of oocysts shed in the feces of the control and inoculated groups. Fecal samples were collected from 6 to 10 days post-infection, and the number of oocysts was determined using a McMaster counting chamber. Total number of oocysts was calculated using the following formula: [total number of oocysts = oocyst count × dilution factor × (fecal sample volume/counting chamber volume)/number of birds per cage]. Body weights were individually measured for 2 weeks before infection and for 10 days post-infection. Differences in mean oocyst production and mean weight gain between the 4 groups were tested by using one-way analysis of variance (ANOVA; GraphPad InStat; GraphPad Software, USA) and considered significant at $p < 0.05$. All studies were performed in accordance with the Guide for Animal Experimentation by Wonkwang University and were approved by the Institutional Animal Care and Use Committee of Wonkwang University (Approval No. WKU11-007). We made all efforts to minimize the pain or discomfort of the animals used in the study.

Fig. 1. The chickens were orally inoculated by gavage by using a 24-gauge stainless-steel animal-feeding tube, which is used for feeding mice, attached to a 3-mL syringe.

As shown in Table 1, oocyst shedding was significantly higher in the inoculated chickens than in the control chickens ($p < 0.05$). The number of fecal oocysts shed was highest on day 7 post-inoculation (Table 1). Moreover, body weight gain was lesser in the animals in the inoculated group than in the animals in the control group (Table 2). Fecal oocyst shedding was observed in the inoculated animals (Fig. 2).

The genus *Eimeria* includes intracellular coccidian (phylum Apicomplexa) parasites that affect the intestinal epithelial cells in chickens. The organisms are pathogenic in the asexual stages of the life cycle, and infection by these organisms clinically manifest as coccidiosis. This parasite is considered to cause the most important poultry infection worldwide [4]. We evaluated the host's susceptibility to avian coccidiosis by enumerating fecal oocysts and determining body weight changes after challenge infection with live coccidial parasites [2, 11]. Thus far, no efficient *in vitro* model is available for propagating all the life cycle stages of *Eimeria*. For the experiment, *Eimeria* oocysts must be maintained by subsequent inoculation in animals every 6 months, and the maintenance is tedious and time-consuming [16]. In

Table 1. Number of oocysts shed in the feces of studied chickens

Group	Oocyst number (× 10^6) / Days post infection				
	6	7	8	9	10
Control	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Inoculated[a]	12 ± 2.71*	58.2 ± 2.94*	22.8 ± 2.15*	6.5 ± 1.78*	1.3 ± 0.67*

[a]Chickens inoculated with live *Eimeria* oocysts in vaccine.
*Significantly different from control chickens ($p < 0.05$).

Table 2. Changes in body weight of studied chickens

Group	Body weight (g) / Days post infection				
	1	3	5	7	10
Control	117.1 ± 2.88	135.3 ± 3.40	169.4 ± 1.96	215.5 ± 2.42	248.7 ± 3.20
Inoculated[a]	114.3 ± 1.83	12.5 ± 2.64[*]	149.8 ± 3.01[*]	176.5 ± 3.03[*]	216.5 ± 3.63[*]

[a]Chickens inoculated with live *Eimeria* oocysts in vaccine.
[*]Significantly different from control chickens ($p < 0.05$).

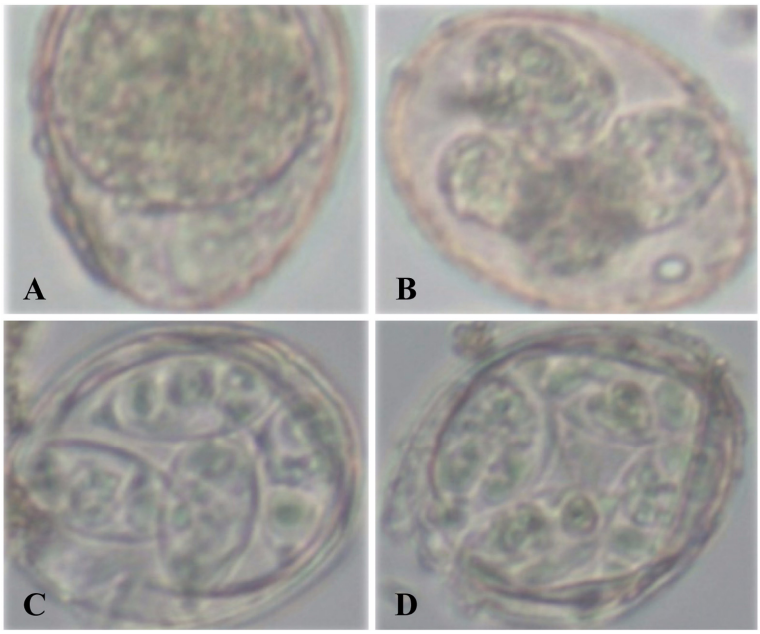

Fig. 2. Fecal oocyst shedding was observed in *Eimeria*-inoculated chickens.

this study, we aimed to develop an animal model system by inoculating animals with a live *Eimeria* vaccine that contains live *Eimeria* oocysts. Various immune system components such as functional activity of T- and/or B-lymphocytes, humoral immune responses, and phagocytes or other cells of the immune system may be adversely affected in the immunosuppressed birds, resulting in suboptimal immune responses to vaccination or increased susceptibility to infections in the host birds [3, 8, 14, 15]. Cyclophosphamide induces humoral immunosuppression by selectively damaging the B-lymphocytes of birds and rats [3, 6, 12, 13]. In this study, all animals were immunosuppressed using cyclophosphamide pretreatment. Thereafter, these immunosuppressed chickens were inoculated with live *Eimeria* oocysts. Fecal oocyst shedding and body weight loss were the most reliable disease parameters and were used to measure the effects of coccidiosis [2, 7]. In this study, significantly greater oocyst shedding was observed in the chickens inoculated with live *Eimeria* oocysts than in the control chickens. The body weights of the control and inoculated group were statistically significant.

On the basis of these results, we suggest that live *Eimeria* vaccine with cyclophosphamide pretreatment may be used to obtain an effective animal model for studying protozoan infections in animals. This animal study model may eliminate the need for a tedious and continuous animal inoculation process every 6 months because live coccidiosis vaccine contains live oocysts.

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