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Evaluation of oocyst shedding of *Eimeria maxima* and *Eimeria acervulina* in broiler chickens

Evaluación del desprendimiento de oocistos de *Eimeria maxima* y *Eimeria acervulina* en pollos de engorde

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ABSTRACT

The purpose of the present study was to evaluate the day and the time of sample collection of an experimental challenge with *Eimeria maxima* (EM) and *Eimeria acervulina* (EA) in broiler chickens. One-day old male Cobb-Vantress broiler chickens were randomly allocated to one of three groups with ten replicates (n=8 chickens/replicate). Chickens were placed in battery cages with a controlled age-appropriate environment: Group 1) Negative control (no challenge or treatment); 2) Challenge control (*Eimeria* challenge only); 3) Challenge + Salinomycin. Challenged chickens were orally gavaged with the mixed culture of EM/EA (10,000 sporulated EM containing 4% wild-type EA) at 14 days of age. Performance parameters were recorded at days 7, 14, 20, and 23. Lesions scores were recorded post-mortem on days 20 and 23. Oocyst per gram (OPG) was performed on days six, seven, and eight post-challenge, and samples were collected at 9:00 AM and 6:00 PM on each day, respectively. Oocyst counts were significantly different ($P < 0.05$) between morning and afternoon on day six post coccidia challenge. The results of this study show that the day and the time at which samples are collected can have a significant effect on the reliability and validity of data.

Keywords: *Eimeria maxima; Eimeria acervulina;* oocysts shedding*;* performance parameters

RESUMEN

El propósito del presente estudio fue evaluar el día y la hora de recolección de la muestra de un desafío experimental con *Eimeria maxima* (EM) y *Eimeria acervulina* (EA) en pollos de engorde. Los pollos de engorde macho Cobb-Vantress de un día de edad se asignaron aleatoriamente a uno de tres grupos con diez réplicas (n = 8 pollos / réplica). Los pollos se colocaron en jaulas de batería con un entorno controlado apropiado para la edad: Grupo 1) Control negativo (sin desafío o tratamiento); 2) Control de desafío (solo desafío Eimeria); 3) Desafío + salinomicina. A los 14 días de edad, los pollos fueron desafiados por vía oral con el cultivo mixto de EM / EA (10,000 EM esporulados que contenían 4% de EA de tipo salvaje). Los parámetros de rendimiento se registraron en los días 7, 14, 20 y 23. Los puntajes de las lesiones se registraron post mortem en los días 20 y 23. El oocisto por gramo (OPG) se realizó en los días seis, siete y ocho después del desafío, y muestras se recolectaron a las 9:00 a.m. y a las 6:00 p.m. de cada día,

respectivamente. Los recuentos de oocistos fueron significativamente diferentes (P < 0.05) entre la mañana y la tarde en el día seis después del desafío con coccidios. Los resultados de este estudio muestran que el día y la hora en que se recolectan las muestras pueden tener un efecto significativo en la confiabilidad y validez de los datos.
Palabras clave: *Eimeria maxima; Eimeria acervulina;* desprendimiento de oocistos; parámetros de rendimiento.

INTRODUCTION

Coccidiosis presently proves to be a major and pressing protozoan disease in the poultry industry worldwide (Dalloul and Lillehoj, 2006). Coccidiosis is caused by a protozoan parasite from the genus *Eimeria*. The life cycle of coccidial parasites includes asexual and sexual replication stages and begins when a bird ingests sporulated oocysts from the environment, as described by Conway and McKenzie (2007). After ingestion, four sporocysts contained in a single sporulated oocyst release two sporozoites. The release of the sporozoites is caused by digestive activity within the chicken. Released sporozoites will then “invade epithelial cells in a specific zone of the intestine or ceca;” which is dependent on the *Eimeria* species (Chapman, 2003). Within the cell, sporozoites become trophozoites and feed for twelve to forty-eight hours to grow and eventually asexually divide via schizogony, or merogony; this stage is known as a schizont or meront. Within the parasite, the merozoite stages form and are released after the schizont matures and ruptures, which takes three days. This first generation of merozoites will invade more epithelial cells and repeat the multiplication process. The second generation of merozoites may induce a third schizogonous cycle; this too is dependent on the *Eimeria* species. Both male (microgametocytes) and female (macrogametocytes) gametocytes will form. Macrogametocytes will grow into macrogametes. Microgametocytes will mature, rupture, and release biflagellate microgametes that fertilize the female macrogametes. Following fertilization, a “thickened wall forms around the macrogamete, forming a zygote” (Conway and McKenzie, 2007; McDougald and Fitz-Coy, 2013). At the conclusion of this cycle, a new oocyst is formed and will pass through the bird’s droppings after rupturing its host cell (Tewari and Maharana, 2011).

Eimeria spp. oocysts, from a single or several simultaneous infections, are excreted in feces over a period of several days. Oocysts shedding starts low, reaches a plateau, and then decreases until the disease runs its course (Clarke, 1979; Williams, 1973). Interestingly, several investigators have reported that oocyst counts differ between morning and evening sampling collections (Hudman *et al*., 2000; Brown *et al*., 2001). This variability has been recognized for several years but has largely been overlooked (Misof, 2004). Recently, however, it has been shown that, the day post-inoculation and the time of day at which samples are collected can have a significant effect on the reliability and validity of the data (Brawner and Hill, 1999). Hence, the purpose of the present study was to evaluate the influence of oocyst shedding variation and day of sampling on experimental challenge with *Eimeria maxima* and *Eimeria acervulina* in broiler chickens.

MATERIALS AND METHODS

Challenge strains

Oocysts of *Eimeria maxima* M6 (EM) and wild-type *E. acervulina* (EA) were provided by Dr. John. R. Barta, University of Guelph, Canada. The methods for detecting and recovering oocysts from infected chickens, oocyst sporulation, and the preparation of infective doses, were conducted as described previously (Haug *et al*., 2006). A dose-titration study was performed to determine the EM/EA coccidia co-challenge dose before starting the experimental trial. At 13 days of age, broilers were weighed, divided into three groups (n = 15/group), and challenged with three different doses (10,000, 20,000, or 40,000) of sporulated oocysts in 1 mL volume by oral gavage. The fourth group of chicks was kept as a negative control. Five days post-challenge, body weight (BW) and body weight gain (BWG) were recorded. In the present study, challenged chickens were orally gavaged at 9:00 am with the mixed culture of EM/EA (10,000 sporulated EM containing 4% wild-type EA) at 14 days of age as this dosage reduced BWG by 35.82%. This is based on the criterion that the challenge dose must cause sub-clinical coccidiosis, consisting of a reduction between 25-35 % in BWG without the presence of clinical signs.

Animal source and experimental design

Two hundred and forty one-day-old male Cobb-Vantress broiler chickens (Fayetteville, AR, USA) were weighed and randomly allocated to one of three groups with ten replicates (n=8 chickens/replicate). Chickens were placed in battery cages, with a controlled age-appropriate environment: Group 1) Negative control (no challenge or treatment); 2) Challenge control (*Eimeria* challenge only); 3) Challenge + Salinomycin at 60 g/ton (Bio-Cox 60, Huvepharma, Peachtree City, GA 30269). Chicks received *ad libitum* access to water and feed for 23 days. An experimental starter diet (Table 1) was formulated to approximate the nutritional requirements of broiler chickens as recommended by the National Research Council (NRC, 1994) and adjusted to breeder's recommendations (Cobb, 2015). Chickens received 23 hours of light from days 1 to 4, 20 hours of light from days 5 to 14, and 18 hours of light from days 15 to 23. Light intensity was set at 30-footcandle the first week, 1-foot candle from days eight to fourteen, and 0.5-footcandle from days 15 to 23. Temperature and light were set to mimic commercial conditions from d 1-21 in all rooms with a gradual reduction on temperature from 32 to 24°C and relative humidity at 55 ± 5%.

Performance parameters: body weight (BW), body weight gain (BWG), feed intake (FI), and feed conversion rate (FCR) were recorded at days 7, 14, 20, and 23. On day 20, half of the chickens from each replicate were weighed and euthanized while the remaining chickens were weighed and euthanized on day 23 in order to evaluate macroscopic lesions according to the scoring system of Johnson and Reid (Johnson and Reid, 1970). Oocyst per gram (OPG) was evaluated on days six, seven, and eight post-challenge, and samples were collected at 9:00 AM and 6:00 PM on each day, respectively. All animal

handling procedures complied with the Institutional Animal Care and Use Committee (IACUC) at the University of Arkansas, Fayetteville. Explicitly, the IACUC approved this study under protocol #21020.

Table 1. Ingredient composition and nutrient content of a corn-soybean starter diet used in all experimental groups on as-is basis.

Item	Starter diet
Ingredients (%)	
Corn	57.34
Soybean meal	34.66
Poultry fat	3.45
Dicalcium phosphate	1.86
Calcium carbonate	0.99
Salt	0.38
DL-Methionine	0.33
L-Lysine HCl	0.31
Threonine	0.16
Vitamin premix[1]	0.20
Mineral premix[2]	0.10
Choline chloride 60%	0.20
Antioxidant[3]	0.02
Calculated analysis	
Metabolizable energy (kcal/ kg)	3,035
Crude protein (%)	22.16
Ether extract (%)	5.68
Lysine (%)	1.35
Methionine (%)	0.64
Methionine + cystine (%)	0.99
Threonine (%)	0.92
Tryptophan (%)	0.28
Total calcium	0.90
Available phosphorus	0.45
Determined analysis	
Crude protein (%)	21.15
Ether extract (%)	6.05
Calcium (%)	0.94
Phosphorus (%)	0.73

[1]Vitamin premix was supplied by the following per kg: vitamin A, 20,000 IU; vitamin D_3, 6,000 IU; vitamin E, 75 IU; vitamin K_3, 6.0 mg; thiamine, 3.0 mg; riboflavin, 8.0 mg; pantothenic acid, 18 mg; niacin, 60 mg; pyridoxine, 5 mg; folic acid, 2 mg; biotin, 0.2 mg; cyanocobalamin, 16 µg; and ascorbic acid, 200 mg (Nutra Blend LLC, Neosho, MO 64850). [2]Mineral was premix supplied at the following per kg: manganese, 120 mg; zinc, 100 mg; iron, 120 mg; copper, 10 to 15 mg; iodine, 0.7 mg; selenium, 0.4 mg; and cobalt, 0.2 mg (Nutra Blend LLC, Neosho, MO 64850). [3]Ethoxyquin.

Data and statistical analysis

Lesions scores, oocyst per gram, and performance data were subjected to ANOVA as a completely randomized design using the GLM procedure of SAS (SAS, 2002). For growth performance parameters (BW, BWG, FI, and FCR), each replicate cage was considered as an experimental unit. Treatment means were partitioned using Duncan's multiple range test at $P<0.05$ indicating statistical significance.

RESULTS AND DISCUSSION

The results of the evaluation of body weight, body weight gain, feed intake, and feed conversion ratio in broiler chickens challenged with coccidia are summarized in Table 2. All three groups started with similar BW; however, at d 7, there was an increase in the BW of chickens treated with Salinomycin. By day 20 (6 days post-challenge), the the negative control group (no challenged or treated) and challenge Salinomycin treated group exhibited a significant increase in BW when compared with the challenged control group ($P < 0.05$). Interestingly, by day 23 (9 days post-challenge), there were only significant differences in BW between the negative control group and challenge control. A similar trend was observed in BWG and FCR. No significant differences were observed in FI among the three groups (Table 2).

Table 3 shows the results of the evaluation of *E. maxima* oocyst per gram count in the feces of broilers on day 6 through day 8 post-challenge at different times of the day, the average per day. Although there was some recovery of oocysts from unchallenged untreated control chickens, there was significantly less OPG in this group compared to both challenge groups. No significant difference in OPG was observed between both challenge groups during the three days of evaluation (Table 3). In the present study, it was remarkable to find that EM oocysts were excreted in very high numbers on day 6 post-challenge in the evening for all three experimental groups. When combining and obtaining the average OPG, day 6 showed a higher number of EM oocysts, and the expected significant differences between the OPG amongst the three experimental groups were observed (Table 3). Similarly, significant differences were found in the lesion scores for EM for both evaluation days (20 and d 23) between the three experimental groups. Nevertheless, a higher number of oocysts were recovered on d 20 in both challenged groups compared to d 23 (Table 3). In the present study, negative control chickens were randomly assigned into the experimental groups that were challenged with coccidia. Perhaps, that is the reason that these chickens showed some infection, due to cross contamination of feces among the cages. Clearly, in future studies, negative control chickens must be placed in a separate room and if this is not possible, separate, and isolated cages.

The results of the evaluation of *E. acervulina* oocyst per gram count in the feces of broilers on day 6 through day 8 post-challenge at different times of the day, the average per day, and lesions scores on days 20 and 23 are summarized on Table 4. A similar trend was observed in the OPG for EA, although more oocysts were present on day 23 in the challenged groups than day 20 (Table 4). Macroscopic intestinal lesions scores on days 20 and 23 of age are showed in table 5. In summary, oocyst counts were significantly different between morning and evening on d 6 post-challenge. The increased shedding of oocysts in the evening samples collections are in accordance with earlier studies of the diurnal excretion of *Eimeria* spp. oocysts (Hudman *et al*., 2000; Brown *et al*., 2001; Misof, 2004).

Coccidiosis remains one of the most critical diseases in poultry and results in the annual loss of millions of US dollars by the poultry industry (Williams, 2005; Chapman, 1999). One common practice in managing coccidiosis is the use of prophylactic drugs and antimicrobials that inhibit the development of sporozoites/ merozoites. However, the poultry industry is now experiencing increasing drug resistance in *Eimeria* strains (Abbas *et al*., 2012). Thus, pressure from decreasing chemical efficacy has increased demand for new treatment methods such as through plant products. As there are clear advantages to an effective controlling agent without complications with *Eimeria* drug resistance, there is merit in searching for effective methods with mechanisms alternative to traditional anticoccidial chemotherapeutics (Naidoo *et al*., 2008; Masood *et al*., 2013). Vaccination against coccidiosis is one alternative to chemical use. When vaccinating against coccidiosis, the natural immune system of the animal is employed to combat potential infections in the future (Shivaramaiah *et al*., 2014). Conventionally, live or attenuated parasites are utilized and *Eimeria* specific vaccines can incorporate multiple species or strains (Shivaramaiah *et al*., 2014). Attenuated *Eimeria* parasites can be selected through “precociousness,” in which “drug-sensitive, virulent strains of *Eimeria* spp.” are allowed to pass through a species host, reproducing, and being developed into attenuated vaccines (Peek and Landman, 2011; Shirley *et al*., 2007).

Previous research has described circadian variation in oocyst shedding across multiple avian host species (Hudman *et al*., 2000; Brown *et al*., 2001). Consequently, if circadian variation in oocyst shedding is not accounted for, the results of such testing are unreliable and may be misleading (Misof, 2004). A suitable method for obtaining accurate data seems to be to restrict the sampling period.

The results of this study show that the day and the time at which samples are collected can have a significant impact on data and reinforces the importance of collecting the fecal samples at the same time of day post-challenge. Oocyst counts were significantly different between morning and afternoon on day six post coccidia challenge. Coccidia load sampling should be restricted to the second half of the total daylight time. This more

restrictive period should thus be considered as the preferred period for obtaining reliable information.

Table 2. Evaluation of body weight, body weight gain, feed intake, and feed conversion ratio in broiler chickens challenged with coccidia.

Item	Negative control (no challenge or treatment)	Challenge control (*Eimeria* challenge only)	Challenge + treated Salinomycin Sodium (60 g/ton)
Body weight (g)			
d 0	46.60 ± 0.19	46.16 ± 0.36	46.70 ± 0.42
d 7	145.05 ± 2.16 ^{ba}	144.48 ± 2.51 ^{ba}	148.95 ± 2.33 ^{a}
d 14	401.30 ± 7.28 ^{bc}	402.63 ± 9.21 ^{bac}	429.16 ± 5.11 ^{a}
d 20	736.14 ± 11.23 ^{a}	671.97 ± 16.35 ^{b}	751.09 ± 8.00 ^{a}
d 23	927.51 ± 20.06 ^{a}	781.25 ± 42.28 ^{b}	881.38 ± 23.87 ^{ba}
Body weight gain (g)			
d 0 to 7	98.45 ± 2.13 ^{ba}	98.31 ± 2.54 ^{ba}	102.25 ± 2.36 ^{a}
d 7 to 14	256.26 ± 6.15	258.16 ± 7.55	280.21 ± 3.82 ^{a}
d 14 to 20	334.84 ± 5.67 ^{a}	269.34 ± 11.69 ^{b}	321.93 ± 5.47 ^{a}
d 0 to 23	890.31 ± 29.18 ^{a}	735.65 ± 42.60 ^{b}	834.03 ± 23.61
Feed intake (g)			
d 0 to 14	609.89 ± 12.51	621.93 ± 16.27	641.06 ± 15.45
d 0 to 20	930.19 ± 37.87	909.81 ± 36.98	770.41 ± 30.53
d 0 to 23	1323.79 ± 31.08	1198.68 ± 57.19	1325.55 ± 29.11
Feed conversion ratio (adjusted)			
d 0 to 14	1.50 ± 0.03	1.53 ± 0.03	1.50 ± 0.04
d 0 to 20	1.44 ± 0.01 ^{b}	1.49 ± 0.02	1.45 ± 0.02
d 0 to 23	1.41 ± 0.06 ^{b}	1.54 ± 0.03	1.51 ± 0.03

Chickens were challenged with *Eimeria maxima* (M6) and *Eimeria acervulina* (wild type) by oral gavage at 14 days. $^{a-c}$ Mean values in the same row that do not share a common letter differ significantly ($P < 0.05$). Each value represents the mean ± standard error. Ten replicates, n = 8.

CONCLUSIONS

The results of this study show that the day and the time at which samples are collected can have a significant impact on data and reinforces the importance of collecting the fecal samples at the same time of day post-challenge. Coccidia load sampling should be restricted to the second half of the total daylight time. This more restrictive period should thus be considered as the preferred period for obtaining reliable information.

Table 3. Evaluation of *E. maxima* oocyst per gram count[1] in the feces of broilers on day 6 through day 8 post challenge at different times of the day and average per day.

Treatment	Day 6 / 9:00 AM (day 20)	Day 6 / 6:00 PM (day 20)	Day 6 Average AM/PM	Day 7 / 9:00 AM (day 21)	Day 7 / 6:00 PM (day 21)	Day 7 Average AM/PM	Day 8 / 9:00 AM (day 22)	Day 8 / 6:00 PM (day 22)	Day 8 Average AM/PM
Negative control	239 ± 123.12$^{b\,z}$	1,880 ± 350.09$^{b\,y}$	1,059 ± 192.37$^{b\,z}$	2,214 ± 2,055.28$^{b\,y}$	465 ± 164.91$^{b\,z}$	1,340 ± 1,080.81$^{b\,z}$	312 ± 247.92$^{b\,z}$	504 ± 399.77$^{b\,z}$	408 ± 319.28$^{b\,y}$
Challenge control	39,756 ± 8,540.64$^{a\,y}$	392,859 ± 53742.38$^{a\,y}$	216,308 ± 26,680.84$^{a\,x}$	258,783 ± 34093.03$^{a\,w}$	73,803 ± 20,753.83$^{a\,x}$	166,293 ± 24,541.92$^{a\,y}$	39,751 ± 10,808.39$^{a\,y}$	12,844 ± 2,256.07$^{a\,z}$	26,298 ± 5888.63$^{a\,z}$
Challenge + treated Salinomycin Sodium	28,060 ± 11,708.46$^{a\,y}$	304,517 ± 31,024.37$^{a\,y}$	166,288 ± 11,708.46$^{a\,y}$	180,752 ± 39,771.21$^{a\,w}$	86,940 ± 22,231.97$^{a\,x}$	133,846 ± 39,771.21$^{a\,y}$	23,440 ± 5,199.72$^{a\,yz}$	11,966 ± 1,207.11$^{a\,z}$	17,703 ± 5199.72$^{a\,z}$

Table 4. Evaluation of *E. acervulina* oocyst per gram count[1] in the feces of broilers on day 6 through day 8 post challenge at different times of the day and average per day.

Treatment	Day 6 / 9:00 AM (day 20)	Day 6 / 6:00 PM (day 20)	Day 6 Average AM/PM	Day 7 / 9:00 AM (day 21)	Day 7 / 6:00 PM (day 21)	Day 7 Average AM/PM	Day 8 / 9:00 AM (day 22)	Day 8 / 6:00 PM (day 22)	Day 8 Average AM/PM
Negative control	83 ± 28.07$^{a\,y}$	52 ± 30.20$^{c\,y}$	55 ± 29.65$^{c\,y}$	0 ± 0$^{c\,z}$	0 ± 0$^{b\,z}$	0 ± 0$^{c\,z}$	52 ± 52.08$^{c\,y}$	26 ± 26.25$^{c\,y}$	52 ± 52.21$^{c\,y}$
Challenge control	1,000 ± 319.03$^{a\,yz}$	5,993 ± 995.74$^{a\,w}$	3,497 ± 352.58$^{a\,y}$	6,656 ± 1924.02$^{a\,w}$	3,007 ± 1266.66$^{a\,x}$	4,831 ± 1580.37$^{a\,y}$	1,800 ± 212.01$^{ab\,y}$	779 ± 228.56$^{ab\,z}$	1,289 ± 182.52$^{ba\,z}$
Challenge + treated Salinomycin Sodium	194 ± 54.9$^{a\,z}$	1,849 ± 498.62$^{b\,w}$	1,022 ± 241.84$^{b\,y}$	2,287 ± 335.90$^{b\,w}$	1,912 ± 514.33$^{a\,w}$	2,099 ± 270.65$^{b\,y}$	1,339 ± 88.24$^{b\,x}$	552 ± 99.01$^{b\,y}$	945 ± 69.81$^{b\,z}$

Chickens were challenged with *Eimeria maxima* (M6) and *Eimeria acervulina* (wild type) by oral gavage at 14 days of age. [1]Each value represents the mean ± standard error. Five replicates/ n = 5. [a-c] Mean values in the same column that do not share a common letter differ significantly. [w-z] Mean values in the same row that do not share a common letter differ significantly ($P < 0.05$). [2]Each value represents the mean ± standard error. Ten replicates/n = 8. [a-c] Mean values in the same column that do not share a common letter differ significantly.

Table 5. Macroscopic intestinal lesions scores[1] on days 20 and 23 of age for broiler chickens challenged with *Eimeria acervulina* and *E. maxima*.

Treatment	*Eimeria acervulina*		*Eimeria maxima*	
	day 20	day 23	day 20	day 23
Negative control	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}	0.05 ± 0.03 ^{c}	0.00 ± 0.00 ^{c}
Challenge control	0.80 ± 0.12$^{a\,z}$	1.45 ± 0.11$^{a\,y}$	1.93 ± 0.10 $^{a\,y}$	1.25 ± 0.08 $^{a\,z}$
Challenge + treated Salinomycin Sodium	0.48 ± 0.11$^{b\,z}$	0.90 ± 0.09$^{b\,y}$	1.28 ± 0.14 $^{b\,y}$	0.77 ± 0.09 $^{b\,z}$

Chickens were challenged with *Eimeria maxima* (M6) and *Eimeria acervulina* (wild type) by oral gavage at 14 days of age. [1]Each value represents the mean ± standard error. Five replicates, n = 5. $^{a\text{-}c}$ Mean values in the same column that do not share a common letter differ significantly. $^{w\text{-}z}$ Mean values in the same row that do not share a common letter differ significantly, (P < 0.05). [2]Each value represents the mean ± standard error. Ten replicates, n = 8. $^{a\text{-}c}$ Mean values in the same column that do not share a common letter differ significantly, (P < 0.05).

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