Koch's Postulate in Reproduction of Broiler Coccidiosis by Co-infection with Eight Most Common *Eimeria spp.*: a Model for Future Evaluation of New Biologics

Elie K. Barbour[1],*

Danielle B. Ayyash[2]

Houssam Shaib[2]

Robert R. Bragg[3]

Esam Azhar[4]

Archana Iyer[5]

Steve Harakeh[4]

Zakaria Kambris[6]

Taha Kumosani[5]

[1]*Animal and Veterinary Sciences Department, Faculty of Agricultural and Food Sciences, American University of Beirut, P.O. Box 11-0236, Beirut, Lebanon; adjunct to Biochemistry Department, Faculty of Science and Experimental Biochemistry Unit, King Fahd Medical Research Center, King Abdulaziz University, Jeddah, Saudi Arabia*

[2]*Animal and Veterinary Sciences Department, Faculty of Agricultural and Food Sciences, American University of Beirut, P.O. Box 11-0236, Beirut, Lebanon*

[3] *Department of Microbial, Biochemical and Food Biotechnology, University of the Free State, Nelson Mandela Drive, Bloemfontein, 9300, South Africa.*

[4]*Special Infectious Agents Unit – Biosafety Level 3, King Fahad Medical Research Center; King Abdulaziz University, Jeddah, Saudi Arabia*

[5]*Biochemistry Department, Faculty of Science and Experimental Biochemistry Unit, King Fahd Medical Research Center, King Abdulaziz University, Jeddah, Saudi Arabia*

[6]*Biology Dept, American University of Beirut*

** Corresponding author: Department of Animal and Veterinary Sciences, Faculty of Agricultural and Food Sciences, American University of Beirut, P.O. Box 11-0236, Beirut, Lebanon, Tel: +961-1-350000, Fax: +961-1-744460, E-mail: eb01@aub.edu.lb*

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ABSTRACT

The purpose of this research is to establish a model of Koch's postulate for reproducing coccidiosis in broilers by co-infection with eight most common *Eimeria spp*. involved in this economic disease, in an attempt to use this model in future evaluation of new controlling biologics. Four groups of broilers each challenged at a different age (14, 21, 28, and 35 d) with an equivalent number of sporulated oocysts of eight *Eimeria spp*. had a reduction in their mean weight gain of 10.2% compared to the four parallel control groups of birds that were deprived of the challenge. The mean feed to live body

Table 1. *Description of the different treatments of broilers*

Groups of Broilers	Number of Broilers	Broiler age at Challenge (d)[1]	Broiler age at sacrification (d)
Challenged	40	_	_
1	10	14	20
2	10	21	27
3	10	28	34
4	10	35	41
Control	40	_	_
1	10	14	20
2	10	21	27
3	10	28	34
4	10	35	41

[1]The esophageal challenge was with a total of $1.76x10^5$ oocysts/bird of equal number of oocysts for all the 8 species of Eimeria

weight conversion ratio increased significantly from 1.5 in the four unchallenged-control groups to 3.1 in the four challenged groups (P<0.05). The mean frequency of mortality increased up to 15% in the challenged groups in comparison to 5% in the controls. This higher mortality was associated in most challenged groups with significant increase in the mean lesion scores and mean oocyst count in the intestine compared to those observed in the controls. The benefit of this established model of Koch's postulate for reproducing coccidiosis in broilers, in future search of new controlling biologics, will be presented.

INTRODUCTION

Coccidiosis is the most important economic protozoan disease affecting poultry industry worldwide, having an annual loss of more than US $ 4 billion (Shirley et al 2004; Williams 1999a). Eighty % losses are due to mortality, reduced weight, and inefficient feed conversion (Dalloul and Lillehoj 2005).

The etiologic agents of coccidiosis are various *Eimeria spp*., which invade the lining of the intestine and are transmitted from bird to bird via the ingested sporulated oocysts found in the environment. The most common coccidial infections in broilers are caused by eight species of Eimeria (Lee et al 2011). These are *E. acervulina* (Assis et al 2010), *E. brunetti* (Hein 1974), *E. hagani* (Joyner and Long 1974), *E. maxima* (Shnitzler and Shirley 1999), *E. mivati* (Vrba et al 2011), *E. necatrix* (Conway and Mckenzie 2007), *E. praecox* (Reperant et al 2012), and *E. tenella* (Railliet and Lucet 1891).

The optimization of Koch's Postulate for reproduction of the intestinal pathogenesis by a controlled challenge with the eight most common species of Eimeria is of paramount importance, before evaluation of any drug or immunopotentiator against these protozoas (Elmusharaf et al 2010). In addition, the multiplication and pathogenesis of *Eimeria spp*. is related to innate immunity of different chicken breeds (Lillehoj 1994); thus, the inclusion of a certain breed in the Eimeria spp. challenge, requires a detailed optimization to reproduce the pathogenesis of the challenging organisms in its birds. The establishment of a chicken model for achieving Koch's postulate, using co-infection by the eight most common species of Eimeria, is of paramount importance in future search for new control programs, and in discovery of biologics that can provide wider leverage in coccidiostat-rotation programs on broiler farms.

To our knowledge, this is the first

Table 2. *Percentage of weight increase in the four control and the four Eimeria-challenged groups after 6 days of assigned challenges*

Group[1] of broilers	Age at assigned challenge (days)	Percentage of weight increase at 6 days period following each assigned challenge		
		Controls	Challenged	SEM[2]
1	14	96.3	88.2	
2	21	69.0	54.5	
3	28	41.2	31.7	
4	35	28.8	20.2	
Mean		58.8[a]	48.6[a]	10.0

[1]Each of the four control and four challenged groups had10 birds
[2]SEM is Standard Error of Means
[a]Means in a row followed by the same alphabet superscript are not significantly different (P>0.05)

research attempting to establish a Koch's postulate for reproducing coccidiosis in broilers by an equivalent number of sporulated oocysts of eight *Eimeria spp*.

MATERIALS AND METHODS

Birds and feed

A total of 80 day-old Ross 308 birds were divided into 8 groups, with 10 birds/group. The birds were fed according to nutrient requirements recommended by the NRC 1992. The feed did not contain any coccidiostat. It is worth noting that the approval of Institutional Animal Care and Use Committee at the Medical School of the American University of Beirut was obtained before the initiation of this study.

Description of the different treatments

The description of the different treatments is presented in Table 1. The birds were divided into 8 groups, with 10 birds/group. The assignment of challenge at a different age (14, 21, 28, and 29 d) to each of the first four groups by the eight *Eimeria spp*. is indicated in Table 1. The four other groups were the parallel controls that were deprived of the Eimeria challenge.

The esophageal challenge was with a total of $1.76x10^5$ oocysts/bird of equal number of oocysts of all eight species of *Eimeria* namely, *E. acervulina*, *E. brunetti*, *E. hagani*, *E. maxima*, *E. mivati*, *E. necatrix*, *E. praecox*, and *E. tenella*.

Evaluation of the Koch's Postulate

The evaluation of the Koch's postulate in reproducing coccidiosis by the co-infection with the eight *Eimeria spp*. in broilers required quantitative assessment of five parameters, observed during and/or at the end of the six days period following each of the four challenge dates. This quantitative assessment was in accordance with previously established protocols for Anti-coccidial Susceptibility Testing (AST) (Holdsworth et al 2004; USDHHS 2012). The measured parameters for comparison of the four challenged to the four control groups included:

1) the mean percent of weight increase
2) the mean feed conversion ratio
3) the mean percent mortality,
4) the mean sore of lesions (0-3 scores) in each of the four intestinal organs (duodenum, jejunum, ileum, and cecum),
5) and the mean of Eimeria oocysts counts per gram of each of the four intestinal organs, observed at the end of the 6 days period following each of the four challenges.

The weight gain was calculated by subtraction of the chicken weight at the end of the 6 days following the challenge from the weight at challenge time. The feed conversion was calculated by the division of the consumed weight of feed by each group of chicken, during the 6 days following the challenge, divided by the birds live weight

Table 3. Feed conversion in the four control and the four Eimeria-challenged groups at 6 days-incubation period following the assigned challenges

Group[1] of broilers	Age at assigned challenge (days)	Feed conversion during 6 days following challenge		
		Controls	Challenged	SEM[2]
1	14	1.1	3.0	
2	21	1.7	3.3	
3	28	2.1	2.5	
4	35	2.1	3.7	
Mean		1.7[a]	3.1[b]	0.3

[1]Each of the four control and four challenged groups had 10 birds
[2]SEM is Standard Error of Means
[a,b]Means in a row followed by different alphabet superscripts are significantly different (P<0.05)

gain during that period. The mortality frequency was calculated during the 6 days period following challenge. The scoring of the lesions was accomplished by a trained researcher, scoring '1', '2', and '3' to respective intestinal lesions of mild, moderate, and severe congestion. The determination of the oocytes count per gram of each of the 4 intestinal organs was according to the protocol documented by Haug and co-workers (2006) and Conway and Mckenzie, (2007). Briefly, the intestine was rinsed in a saline solution to remove the fecal material, and then weighed. The weighed intestinal organs was homogenized and put in a 35% NaCl solution for the floatation of the oocytes. The floated oocytes were counted in a McMaster chamber and the data reported the count per gram of the sampled intestine.

Statistics

The mean of the above measured parameters were compared among the different treatments by ANOVA followed by Tukey's test, reporting significant differences in means at P<0.05. The mortality frequencies were compared among the different treatments by the CHI Square method.

RESULTS AND DISCUSSION

Table 2 shows the percent increase in weights, during the six days-incubation period of the *Eimeria spp*., in the four control groups and the four groups of birds challenged at different respective ages of 14, 21, 28, and 35 days old.

All groups of birds challenged at different ages had a lower percentage of weight increase during the 6 days-incubation period compared to their respective control-unchallenged groups. This reduction in mean weight gain by the challenged group in comparison to controls was equivalent to 10.2%. The mean % weight increase of all control groups didn't differ statistically from the challenged groups (P>0.05), in spite of the consistent trend of reduction in % weight gain by the challenged compared to control groups. This is mainly due to the magnitude of the Standard error of means (SEM). However, this reduction of around 10 % in weight gain by challenged birds is of great significance in the economy of intensive broiler production (Cahaner and Leenstra 1992; Havenstein et al 2003).

Table 3 shows the comparison of the mean feed conversion in the four control versus the four challenged groups. There was a consistent higher feed conversion to live weight in challenged groups compared to their respective controls, with a statistically higher mean conversion in challenged groups compared to controls (P<0.0.5). This inefficiency in feed conversion to weight by the challenged groups is most likely due to the pathogenesis of the *Eimeria spp*. used in the challenge on the intestine, affecting negatively the feed digestion (Assis et al 2010; Major et al 1978). The feed conversion parameter is of paramount importance in this Koch's postulate model that can statisti-

Table 4. *Frequency of mortality in the four control and the four Eimeria-challenged groups at 6 days period following the assigned challenges*

Group[1] of broilers	Age at assigned challenge (days)	Feed conversion during 6 days following challenge		
		Control	Challenged	SEM[2]
1	14	1 (10%)[a]	4 (40%)[a]	
2	21	1 (10%)[a]	2 (20%)[a]	
3	28	0 (0%)[a]	0 (0%)[a]	
4	35	0 (0%)[a]	0 (0%)[a]	
Mean		0.5 (5%)[a]	1.5 (15%)[a]	0.5

[1]Each of the four control and the four Eimeria challenged groups had 10 birds
[2]SEM is Standard Error of Means
[a]Frequencies in a row followed by the same superscript are not significantly different (P>0.05)

cally differentiate the performance between controls and the birds that were challenged with *Eimeria spp*. (Havenstein et al 2003; Williams 1999). In another words, each bird in the challenged groups has to eat an average of 3.1 Kg of feed to convert it to 1 Kg of live weight, while each bird in the controls has to eat only 1.7 Kg of feed to convert it to 1 Kg of live weight. This significant inefficiency in converting feed to live weight in challenged birds has a great negative impact in economic losses in intensive poultry production operations (Williams 1999).

Table 4 presents the data related to frequency of mortality in the control and challenged groups during the 6 days-incubation period following the assigned challenges. The mortality in the groups challenged at 14 and 21 days of age was higher than their corresponding control groups. However, there was no mortality in groups of birds challenged at 28 and 35 days of age neither in their corresponding control groups, which resulted in insignificant difference in the mean frequency of mortality between all control and challenged groups (P>0.05). The experimental design seems to create mortality when the Eimeria challenge is administered at earlier age of 14 and 21 days of age but not in older birds (Brackett & Bliznick 1952). The trend in getting higher mortality in birds challenged with this cocktail of *Eimeria spp*. at 14 and 21 days of age compared to their corresponding controls will be useful in future evaluation of new control programs including the search for new biologics to protect against coccidiosis in broilers.

Table 5 compares the gross lesions score in different parts of the intestine of the four control and the four groups of broilers challenged at different ages, using Johnson & Reid (1970) method. The mean score of the lesions in different parts of the intestine of challenged birds was always higher in the groups of birds administered the challenge at earlier age of 14 and 21 days old compared to birds challenged at older ages of 28 and 35 days. This data correlates with the mortality data shown in Table 4, in which birds challenged at older ages of 28 and 35 days had no mortality (Chapman et al 2005; Long et al 1980; Ramsburg et al 2003). However, these milder lesions in the two groups of broilers challenged at respective older ages of 28 and 35 days seem to raise the inefficiency in feed conversion of these birds (Table 3). In addition, most of the examined intestinal parts had higher significant lesion scores in birds challenged at 14, 21, and 28 days of age compared to the same intestinal parts of their corresponding control groups (P<0.05). This significant difference in mean lesion score of the different parts of the intestine didn't exist between the group of birds that was challenged at 35 days of age compared to its corresponding control group (P>0.05).

Table 6 presents the data related to mean oocyst counts in different intestinal parts of

Table 5. Mean score of lesions in different parts of the intestine in the four control and the four Eimeria-challenged groups at the end of the 6 days-incubation period following the assigned challenges

Group[1] of broilers	Age at assigned challenge (days)	Intestinal part	Mean score of lesions at 6 days period following each challenge		
			Controls	Challenged	SEM[2]
1	14	Duodenum	0.0^{a}	1.5^{b}	0.25
		Jejunum	0.0^{a}	1.1^{b}	0.18
		Ileum	0.0^{a}	1.6^{b}	0.25
		Cecum	0.0^{a}	1.1^{b}	0.21
2	21	Duodenum	0.2^{a}	2.0^{b}	0.27
		Jejunum	0.0^{a}	1.7^{b}	0.25
		Ileum	0.1^{a}	1.6^{b}	0.26
		Cecum	0.2^{a}	1.6^{b}	0.25
3	28	Duodenum	0.6^{a}	1.4^{a}	0.25
		Jejunum	0.3^{a}	1.6^{b}	0.25
		Ileum	0.0^{a}	0.6^{b}	0.15
		Cecum	0.0^{a}	0.9^{b}	0.22
4	35	Duodenum	0.25^{a}	0.5^{a}	0.18
		Jejunum	0.0^{a}	0.38^{a}	0.14
		Ileum	0.0^{a}	0.38^{a}	0.14
		Cecum	0.0^{a}	0.13^{a}	0.06

[1] Each of the four control and the four challenged groups had 10 birds.
[2]SEM is Standard Error of Means
[a,b]Means of lesion scores in a row followed by different alphabet superscripts are significantly different (P<0.05). Lesion scores ranged between 0 to 3.

controls versus groups of birds challenged at different ages. The four control groups kept free from oocyst infection until the end of the six days period following the assigned challenges. This fact proved the efficiency of management followed in the strict isolation units used for rearing the eight groups of birds. The mean oocyst counts was always significantly higher in all examined intestinal parts of the challenged groups compared to zero counts observed in their corresponding controls (p<0.05) (Ogbe et al 2009), except in the group challenged at 35 days of age, in which only the jejunum organ maintained a significantly higher mean oocyst count compared to the mean jejunal oocyst count of the corresponding control birds.

In conclusion, the Koch's Postulate for reproducing coccidiosis in broilers, by the 8 most common *Eimeria spp*. involved in this economic disease, resulted in significant statistical differences in most measured parameters between controls and challenged birds, including the feed conversion, lesion scores and oocyst counts in different parts of the intestine. In addition, and in spite of the insignificant differences in % weight gain and mortality frequency between controls and challenged groups, still this nature of challenge resulted in a trend of reduction in weight gain and in augmentation of mortality in challenged birds compared to controls. These significant differences in identified parameters, and the trend in differences of the other parameters, will allow for future development of new immuno-modulators

Table 6. *Mean oocysts count per gram of intestinal part[1] in the four control and the four Eimeria-challenged groups at the end of 6 days-incubation period following the assigned challenges*

Group[1] of broilers	Age at assigned challenge (days)	Intestinal part	Mean oocyst count per gram of intestinal organ at end of 6 days period following the assigned challenges		
			Controls	Challenged	SEM[2]
1	14	Duodenum	0^a	42254^b	9744
		Jejunum	0^a	67839^b	16177
		Ileum	0^a	112843^b	35342
		Cecum	0^a	96666^b	26708
2	21	Duodenum	0^a	33715^b	8584
		Jejunum	0^a	36636^b	9343
		Ileum	0^a	41896^b	10173
		Cecum	0^a	48041^b	14776
3	28	Duodenum	0^a	6578^b	1438
		Jejunum	0^a	12321^b	3075
		Ileum	0^a	19029^b	4566
		Cecum	0^a	7309^b	1978
4	35	Duodenum	0^a	15344^a	6956
		Jejunum	0^a	6506^b	1569
		Ileum	0^a	2912^a	921
		Cecum	0^a	2367^a	756

[1]A length of 1 cm was cut from the middle of each intestinal organ
[2]Each of the four control and four challenged groups had 10 birds
[a,b] Means of Oocysts count in a row followed by different alphabet superscripts are significantly different (P<0.05).

and coccidiostats, aiming to counter the huge negative economic impact of coccidiosis in the broiler industry.

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REFERENCES

1. Assis RC, Luns FD, Beletti ME, Assis RL, Nasser NM, Faria ES, Cury MC. Histomorphometry and macroscopic intestinal lesions in broilers infected with Eimeria acervulina. *Vet Parasitol*. 2010; 168:185-189.
2. Brackett S, Bliznick A. The relative susceptibility of chickens of different ages to coccidiosis caused by Eimeria necatrix. *Poult Sci*. 1952; 31(1):146-148.
3. Cahaner A, Leenstra F.. Effects of high temperature on growth and efficiency of male and female broilers from lines selected for high weight gain, favorable feed conversion, and high or low fat content. *Poult Sci*. 1992; 71(8):1237-50.
4. Chapman HD, Matsler PL, Muthavarapu VK, Chapman ME. Acquisition of immunity to Eimeria maxima in newly hatched chickens given 100 oocysts. *Avian Dis*. 2005; 49:426-429.
5. Conway DP, Mckenzie ME. Poultry Coccidiosis: diagnostics and testing procedures, 3rd ed. Blackwell Publishing Professional, Ames, IA, USA, 2007:pp. 1-164.
6. Dalloul RA, Lillehoj HS. Recent advances in immunomodulation and vaccination strategies against coccidiosis. *Avian Dis*. 2005; 49(1):1-8.
7. Elmusharaf MA, Mohamed HE, Alhaidary A, Beynen AC. Efficacy and characteristics of different methods of coccidiosis infection in broilers chickens. *Am. J. Anim. Vet Sci.*. 2010; 5:45-51
8. Havenstein GB, Ferket PR, Qureshi MA. Growth,

Livability, and Feed Conversion of 1957 Versus 2001 Broilers When Fed Representative 1957 and 2001 Broiler Diets. *Poult Sci.* 2003; 82:1500-1508.

9. Haug A, Williams RB, Larsen S. Counting coccidial oocysts in chicken faeces: a comparative study of a standard McMaster technique and a new rapid method. *Avian Pathol*. 2006; 136:233-242.
10. Hein H.. Eimeria brunette: Pathogenic effects in young chickens. *Exp Parasitol.* 1974; 36:333-341.
11. Holdsworth PA, Conway DP, McKenzie ME, Dayton AD, Chapman HD, Mathis GF, Skinner JT, Mundt HC, Williams RB. World Association for the advancement of veterinary parasitology (WAAVP). Guidelines for evaluating the efficiency of anticoccidial drugs in chickens and turkeys. *Vet Parasitol.* 2004; 121:189-212.
12. Joyner LP, Long PL. The specific characters of the Eimeria, with special reference to the coccidia of the fowl. *Avian Pathol*. 1974; 3(3):145-157.
13. Johnson J, Reid WM. Anticoccidial drugs: lesion scoring techniques in battery and floor-pen experiments with chickens. *Exp Parasitol*. 1970; 28:30-36.
14. Lee KW, Lillehoj HS, Jang SI, Li GX, Bautista DA, Phillips K, Ritter D, Lillehoj EP, Siragusa GR. Effects of coccidiosis control programs on antibody levels against selected pathogens and serum nitric oxide levels in broilers chickens. *J App Poult Res.* 2011; 20:143-152.
15. Lillehoj HS. Analysis of Eimeria acervulina-induced changes in the intestinal T lymphocyte subpopulations in two chicken strains showing different levels of susceptibility to coccidiosis. *Res Vet Sci.* 1994; 56:1-7.
16. Long PL, Johnson J, Wyatt RD. Eimeria tenella: Clinical effects in partially immune and susceptible chickens. *Poult. Sci.* 1980; 59(10):2221-2224.
17. Major JR Jr, Ruff MD. *Eimeria spp*.: influence of coccidia on digestion (amylolytic activity) in broiler chickens. *Exp Parasitol*. 1978; 45(2):234-240.
18. NRC. The use of drugs in food animals. Benefits and risks. Board on Agriculture, Food and Nutrition Board, Institute of Medicine, National Academy Press, Washington D.C. 1999; pp. 88-109.
19. Ogbe AO, Atawodi SE, Abdu PA, Sannusi A, Itodo AE.. Changes in weight gain, faecal oocyst count and packed cell volume of Eimeria tenella-infected broilers treated with a wild mushroom (Ganoderma lucidum) aqueous extract. *J S Afr Vet Assoc*. 2009; 80(2):97-102.
20. Railliet A, Lucet A. Note sur quelques éspèces de coccidies encore peu étudiees. Bulletin de la Société Zoologique de France. 1891;16:246-250.
21. Reperant J, Dardi M, Pages M, Thomas-Henaff M. Pathogenicity of Eimeria praecox alone or associated with Eimeria acervulina in experimentally infected broiler chickens. *Vet Parasitol*. 2012; 187:333-336.
22. Ramsburg E, Tigelaar R, Craft J, Hayday A. Age-dependent requirement for gamma delta T cells in the primary but not secondary protective immune response against an intestinal parasite. *J Exp Med*. 2003; 198:1403-1414.
23. Shirley MW, Smith AL, Tomley FM. The biology of avian Eimeria with an emphasis on their control by vaccination. *Adv Parasitol*. 2005; 60:285-330.
24. Schnitzler BE, Shirley MW. Immunological aspects of infections with Eimeria maxima: a short review. *Avian Pathol*. 1999; 28:537-543.
25. USDHHS (United States Department of Health and Human Services), FDA, Center for Veterinary Medicine. Guidance for Industry. Evaluating the effectiveness of anticoccidial drugs in food-producing animals. Guidance #217. 2012; Pp. 1-31.
26. Vrba V, Poplestein M, Pakandl M. The discovery of the two types of small subunit ribosomal RNA gene in Eimeria mitis contests the existence of *E. mivati* as an independent species. *Vet Parasitol*. 2011; 183:47-53.
27. Williams RB. A compartmentalised model for the estimation of the cost of coccidiosis to the world's chicken production industry. *Int J Parasitol*. 1999; 29(8):1209-1229.