

Chicken I-FABP as biomarker of chicken intestinal lesion caused by coccidiosis

U Cahyaningsih¹, A S Satyaningtijas², R Tarigan³ and A B Nugraha⁴

^{1,4}**Department** of Animal Diseases and Veterinary Public Health, Faculty of Veterinary Medicine, Bogor Agricultural University

^{2,3}**Department** of Anatomy, Physiology and Pharmacology, Faculty of Veterinary Medicine, Bogor Agricultural University

E-mail: umicahyaningsih@yahoo.co.id

Abstract. Coccidiosis is an infection of the gastrointestinal tract caused by *Eimeria* spp, which causes morbidity, mortality, decreased body weight, and decreased production. Until now, difficulties for monitoring health status because no method for early detection has been found. Intestinal - Fatty Acid Binding Proteins (I-FABP) are intracellular proteins that play a role in the transport and metabolism of long-chain fatty acids. The FABP protein family can be used as a marker for specific tissue damage. I-FABP has never been done as a coccidiosis biomarker in poultry. This study aims to know the concentration of I-FABP in serum from chicken blood infected with various doses of *Eimeria* oocysts. A total of 400-day old chick were divided into four treatment groups: doses of 500 oocysts per ml (D1), 5,000 oocysts per ml (D2) and 50,000 oocysts per ml (D3), and Negative Controls (NC). The parameters used were the number of oocyst, lesion score in chicken intestine and measurement of I-FABP level. The results of this study obtained *Eimeria* oocyst production began out seven days after infection in the stool. The lesion score began to appear on day five after infection with a dose of 5,000 and 50,000 oocysts per chicken, while I-FABP concentrations were detectable on day 2 (48 hours) after infection. The conclusions of this study I-FABP can be used as biomarkers of early detection of gastrointestinal damage.

1. Introduction

Coccidiosis in caecum is a parasitic infection of the genus *Eimeria tenella* which causes inflammation and bloody diarrhea. These parasites exist throughout the world but are more common in tropical and hot tropical countries such as Indonesia and an important disease in chicken farms [1]. Infection with 1000-3000 sporulated *Eimeria tenella* oocysts can cause bloody diarrhea, decreased body weight, decreased feed conversion, death [2]. Factors affecting pathogenicity due to *Eimeria* infection are the amount of oocyst ingested, *Eimeria* species, environmental factors (temperature, oxygen, and humidity), chicken age and feed quality provided [3]. Losses due to this disease lead to death, decreased body weight and egg production. Also, the economic losses in the United States are estimated to reach 1.5 billion US \$, including death, weight loss, and rising medical costs due to this disease [4].

FABP (Fatty Acid Binding Protein) is an intracellular protein and is cytoplasmic found in organs with high metabolic rate such as heart, muscle, intestine, liver, brain, and heart. The organs use fatty acids as a source of aerobic energy [5], [6]. FABP will bind to free fatty acids that can be transported to the cells by diffusion through the outer membrane of mitochondria and then used as an energy



source. The protective function of FABP is by binding long-chain fatty acids and their metabolites that have adverse and lethal effects on cells. FABP then directs fatty acids to peroxisomes to be degraded through activation of Peroxisome Proliferator-Activated Receptors (PPARs) [5-7]. In humans, H-FABP detection has been used as a method to measure the severity of myocardial infarction. This FABP can be used as a biomarker for organ damage. The FABP protein family can be used as a marker for specific tissue damage. I-FABP has never been done as a coccidiosis biomarker in poultry. I-FABP concentration will increase when damage occurs, at that time intestinal crypta condition is still intact. I-FABP occurs immediately in the blood and urine shortly after intestinal damage. In mice, I-FABP levels rise 30 minutes after intestinal arterial occlusion [8],[9]. It shows I-FABP can be used as a biomarker to detect intestinal damage at a very early stage (subclinical) and can be detected in feces, urine, plasma, and serum [5]. I-FABP is a stable, very soluble molecule at room temperature and can be released in the blood and urine after tissue damage, so it can be analyzed using ELISA several hours after taking a sample [10]. Measurements of I-FABP concentrations with ELISA proved to be specific and sensitive for measuring the level of an intestinal mucosal lesion with a specificity and sensitivity level above 90 percent. The use of I-FABP as a diagnostic marker in patients with necrotic enterocolitis and proven to be sensitive to detect intestinal damage of phase 1 (early intestinal damage). I-FABP gene is also present and expressed in chicken enterocytes [11]. The use of I-FABP as a biomarker has been extensively studied in humans, pigs, and cattle and has been shown to detect intestinal damage at an early stage. Research on the use of I-FABP as a biomarker in poultry has never been done although it has been confirmed its existence in the poultry.

Clinical symptoms *E. tenella* is bleeding in cecum that usually comes out with feces on the fifth and sixth day after infection. Clinical symptoms *E. tenella* there are several stages, namely the first stage there are spots on the part of the caecum wall and the contents of the cecum is still normal. The second stage more and more, and already seen bleeding on the caecum, began to occur thickening on the cecum wall, the contents of the cecum are still normal. The third stage is the enlarged caecum, full of clotted blood, the contents of the caecum attached to the mucosa. The fourth stage of the caecal wall becomes thick, the contents of the caecum are detached from the mucosal wall, and the outside is visible red spots which then become white. Meanwhile, clinical symptoms are the chickens become weak, decreased appetite, always gathered, anemia, the highest death between the fourth and sixth days [3].

Currently for monitoring of gastrointestinal damage was done by examining clinical symptoms, hematology, measuring antibody titers for various pathogenic agents, isolating pathogenic agents, examination of feces for the detection of worms and protozoa [12]. Because it detects the digestive tract when it shows clinical symptoms and incurs costs for treatment. If lesions of the gastrointestinal tract in the early stages can be known, then the breeder does not suffer economic losses due to decreased body weight and death.

This study aims to measure the concentration of I-FABP in serum taken from chicken blood at various stages of intestinal damage (subacute, acute, and chronic). If I-FABP can be detected earlier in the serum of blood than in the presence of gastrointestinal damage seen from lesion score, then I-FABP is prospective for biomarker without having to wait for the death of chickens due to gastrointestinal damage that causes bloody diarrhea. If the rapid detection method can be done, then the breeder can detect the health of the digestive tract of livestock quickly like through feces or with a drop of blood.

2. Materials and methods

2.1. Propagation of oocysts *E. tenella* from chicken feces as an infection material

A collection of chicken stool samples from the field suspected of being infected with coccidiosis. Positive stool samples containing *Eimeria*'s oocysts were infected in 100 chickens, then collected oocysts from the *Eimeria*-infected caecum.

2.2. Purification and sporulation oocysts *E. tenella* as an infection material.

Oocysts of chicken caecum were purified by using a solution of saturated salt. Oocysts resulted from purification performed sporulation by addition of 2.5% potassium bichromate, then left at room temperature for three days or until the oocysts were sporulated. Sporulated oocysts are used as an infectious agent.

2.3. Treatment

The number of chickens used was 400 chickens, aged 7 days divided into four treatments: chicken infected with oocyst *E. tenella* with doses of 500 oocysts per ml (D1), 5,000 oocysts per ml (D2), 50,000 oocysts per ml (D3) and Negative Control (NC). Each treatment by infected with *E. tenella* oocysts were conducted in 100 chickens, per oral. Treated chickens were feed without coccidiostat.

2.4. Parameters

2.4. 1. *Calculating oocysts production by Mc Master Method.* Mc Master method is a method to calculate Oocysts Per Gram stool (OPG) by weighing stool as much as 2 gram, then added 58 ml saturated salt as float and homogenized solution. The mixture is taken with a pipette and inserted into the Mc Master count chamber to calculate the oocyst under a microscope with magnification 100 times. The formula used with the McMaster count chamber is:

$$OPG = \frac{n}{V_t} \times \frac{V_t + V_l}{V_{kh}}$$

Notes:

N : Number of calculated oocysts

V_t : The volume of the stool

V_p : Volume of the float solution

V_{kh}: Volume from Mc Master count chamber

2.4.2. *Measuring lesion score on caecum.* Measuring the lesion score of the caecal lumen by performing the necropsy in chickens ranging from 1 to 9 days after infection. **Degree of lesion were scored from 1 (light) – 4 (severe).**

2.4.3. *Measuring I-FABP levels in chicken serum.* ELISA plate has been pre-coated with antibodies specific to Chicken I-FABP. **Chicken serum was done by blood collecting using 1 ml syringe,** inserted in a test tube and then left in a tilted position to collect serum. Measurement of Chicken I-FABP concentration in serum using Chicken I-FABP ELISA kit (Elabscience # E-EL-Ch0802) with a sensitivity of 0.188 ng/ml. ELISA plate has been pre-coated with antibodies specific to Chicken I-FABP. Standard solution of 100 µl or serum is added in ELISA plate, then incubated at 37°C for 90 min. The contents of the plate are then removed and added/inserted Biotinylated detection antibody (1: 100) as much as 100 µl, then incubated at 37°C for 90 minutes. The contents of the plate then discarded and washed as much as three times with 350 µl washing buffer. The HRP conjugate (Horseradish peroxidase 1: 100) was then added as much as 100 µl, then incubated at 37°C for 30 minutes. The TMB substrate was then added as much as 90 µl and incubated at 37°C for 15 minutes. The presence of Chicken I-FABP is characterized by a change in the solution to blue. A stop solution added of 50 µl will change the solution to yellow, then read its absorbance at 450 nm wavelength with ELISA reader pre-coated with antibodies specific to Chicken I-FABP.

2.5. Data analysis

Oocysts production data, I-FABP levels, and lesion score were analyzed by Analysis of Variance (ANOVA) followed by Duncan Multiple Range Test (DMRT).

3. Results and discussion

In this study the production of oocysts at doses of 5,000 (D2) and 50,000 oocysts per chicken (D3) oocysts out steel we seen at day seven after infection, while at dose 500 (D1) was seen on day eight after infection. The treatment groups D2 and D3, on days 7–9 after infection showed increased oocyst production according to the dose of infection. However, on day ten after infection, a dose of 50,000 oocysts per chicken (D3) still shows high oocyst production (table 1).

Table 1. Production of *Eimeria tenella* oocysts in the feces.

No	Treatment Group	Day (after infection) and the number of oocyst									
		1	2	3	4	5	6	7	8	9	10
1	NC	0	0	0	0	0	0	0	0	0	0
2	D1	0	0	0	0	0	0	0	2.300	6.400	400
3	D2	0	0	0	0	0	0	6.700	13.300	18.700	200
4	D3	0	0	0	0	0	0	76.000	64.400	12.900	6.800

Notes: after infection

NC: Negative Control, D1: Chickens infected with *E.tenella* at 500 oocysts per ml, D2: dose of 5,000 oocysts per ml, D3: at 50,000 oocysts per ml

The presence of intestinal inflammation is indicated by pathological changes in the gut that can be assessed by lesion score. The results of this study, revealed the lesion score began to be seen on day five after infection at doses of 5,000 and 50,000 oocysts per chicken (table 2). The lesion score is rather high at a dose of 50,000 oocysts per chicken.

Table 2. Lesion score on chicken cecum after infected *Eimeria tenella*.

No	Treatment Group	Day (after infection) and lession score								
		1	2	3	4	5	6	7	8	9
1	NC	0	0	0	0	0	0	0	0	0
2	D1	0	0	0	0	0	0	0	0	0.33
3	D2	0	0	0	0	0.67	1.33	0.3	0.33	0.33
4	D3	0	0	0	0	1.67	2	1.33	2.33	1.33

Notes: after infection

NC: Negative Control, D1: Chickens infected with *E.tenella* at 500 oocysts per ml, D2: dose of 5,000 oocysts per ml, D3: at 50,000 oocysts per ml.

Damage on chicken cecum occurs earlier than oocysts production. Based on the *Eimeria* life cycle on day 5, the ruptured schizont stage removes merozoites, causing intestinal damage and bleeding. After the schizont ruptures, there will be a gametogoni stage that produces the oocyst in the intestine that will come out with the feces on the 7th day after the infection.

I-FABP is an intracellular protein found in gastric epithelial cells, small intestine mucosa, large intestine and represents 2 percent of total enterocyte-produced protein [13]. I-FABP plays a role in mediating lipid metabolism and is also involved in intestinal inflammation through the modulation of critical lipid-sensitive pathways in macrophages and adipose tissue. I-FABP also plays an important role in maintaining the integrity of intestinal epithelial barrier. Damage to the gastrointes-tinal membrane resulting from oocyst infections *E. tenella* leads to the release of intracellular proteins, one of which I-FABP to the extracellular (blood flow) resulting in increased concentration of I-FABP in

the bloodstream and can serve as a biomarker marker of gastrointestinal damage. The concentration of chicken i-FABP from chicken serum infected with *E. tenella* is presented in table 3.

Table 3. Concentration of Chicken I-FABP in treatment group after *E.tenella* infection.

Treatment Group	Chicken I-FABP concentration (ng / ml)					
	hour (after infection)					
	0	48	84	108	120	144
NC	0.00 ± 0.00	0.00 ± 0.00	0.00± 0.00	0.30 ± 0.11	0.67 ± 0.75	0.57± 0.47
D1	0.00 ± 0.00	0.52 ± 0.26	2.54 ± 1.18	1.96 ± 0.90	2.26 ± 0.11	1.46 ± 0.26
D2	0.00 ± 0.00	1.86 ± 0.84	2.14 ± 1.29	2.20 ± 0.67	2.13 ± 0.46	2.40 ± 1.98
D3	0.00 ± 0.00	0.13 ± 0.05	1.72 ± 0.12	2.39 ± 1.09	1.71 ± 0.71	2.42 ± 0.50

Notes: after infection

NC: Negative Control, D1: Chickens infected with *E.tenella* at 500 oocysts per ml, D2: dose of 5,000 oocysts per ml, D3: at 50,000 oocysts per ml.

Data on Table 2 showed that I-FABP can be detected in serum 48 hours after infection in treatment groups D1, D2, and D3. It occurs much more rapidly than the lesions of the gastrointestinal tract seen at five days (120 hours) after infection through anatomical pathology examination. The presence of digestive tract damage in this study was indicated by clinical symptoms of bloody diarrhea and can result in death.

The concentration of I-FABP increased almost doubled at 84 hours after infection. This suggests that gastrointestinal wall damage is continuing and result in an increased amount of I-FABP in the serum. However, the concentration of I-FABP did not increase significantly after 84 h post-infection. This is evident from the increase in I-FABP concentrations that are not comparable with the dose of infection, the number of oocysts and the severity of lesion score. In the case of abdominal injury in humans, the concentration of I-FABP will increase up to 6 hours post trauma, then decrease its concentration [10]. In the induction of ischemia in pigs through the binding of *A. centenaries*, the concentration of I-FABP will increase two h post-ligation and decrease its concentration after reperfusion [14]. This indicates that the concentration of I-FABP will increase rapidly when intestinal damage occurs long before the onset of clinical symptoms. One of the factors causing normal I-FABP (decrease in I-FABP concentration) is a restoration of blood flow to the intestine with membrane integrity recovered so that I-FABP leak can be prevented. In this research, the decrease of I-FABP concentration is also caused by the self-limiting disease.

4. Conclusion

Based on the results of oocyst production and lesions score due to *E. tenella* infection (coccidiosis), I-FABP can be detected as early as 48 hours after infection, before the occurrence of digestive tract damage (5 days after infection). This study suggested, I-FABP can be used as an early detection biomarker of gastrointestinal tract damage.

References

- [1] Allen P C, Fetterer 2002 Recent advances in biology and immunology of *Eimeria* species and diagnosis and control of infection with these coccidian parasites of poultry *J of Clinical Microbiology Micro.* **15** 58–65
- [2] Mc Dougald L R and Reid W M 1997 Coccidiosis, pp 865–883. In *Disease of Poultry* 10th Edition Calnek BW, ed. IOWA State Univ Pr.Ames, IA
- [3] Kennedy MJ. 2001. Coccidiosis in cattle. In: AGRIFACTS Edmonton, Alberta, Canada Alberta Agriculture, Food and Rural Development, Government of Alberta

- [4] Lee, JT, Burke, Anderson, Caldwell 2009 Evaluation of live oocysts vaccination or Salinomycin for control of field-strain *Eimeria* challenge in broiler on two different feeding programs *J. Appl. Poult. Res.* **18** 458–464
- [5] Glatz JFC, Van der Vusse JG 1997 Cellular fatty acid-binding proteins: Their function and physiological significance *Prog. Lipid Res.* **35**(3) 243–282
- [6] Glatz JF, Storch J 2001 Unravelling the significance of cellular fatty acid-binding proteins *Cur Opin Lipidol.* **12** 267–74
- [7] Wolfrum C, Borrmann CM, Borchers T, Spener F 2001 Fatty acids and hypolipidemic drugs regulate peroxisome proliferator-activated receptors α - and γ -mediated gene expression via liver fatty acid binding protein: a signaling path to the nucleus *Proc Natl Acad Sci USA.* **98** 2
- [8] Pelsers MAL, Namiot Z, Kisielewski W, Namiot A, Januszkiewicz M, Hermens WT, Glatz JFC 2003 Intestinal-type and liver-type fatty acid-binding protein in the intestine. Tissue distribution and clinical utility *Clinical Biochemistry.* **36** 529–535
- [9] Pelsers MAL, Hermens WT, Glatz JFC 2005 Fatty acid-binding proteins as plasma markers of tissue injury *Clinica Chimica Acta.* **352** 15–3
- [10] Relja B, Szermutzky M., Henrich D, Maier M, De Haan J, Lubbers T, Buurman WA, Marzi I 2010 Intestinal-FABP and Liver-FABP: Novel Markers for Severe Abdominal Injury *Academic Emergency Medicine.* **17** 729–735
- [11] Wang Q, Hui L, Liu S, Wang G, Wang Y 2011 Cloning and Tissue Expression of Chicken Heart Fatty Acid-Binding Protein and Intestine Fatty Acid Binding Protein Genes *Animal Biotechnology.* **16** 191–201
- [12] Kosek M, Haque, R, Lima, A, Babji S, Shrestha S, Qureshi S, Amidou S, Mduma E, Lee G, Yori PP, Guerrant RL, Bhutta Z, Mason C, Kang G, Kabir M, Amour C, Bessong P, Turab A, Seidman J, Olortegui MP, Lang D, Gratz J, Miller M, Gottlieb M 2013 Fecal Markers of Intestinal Inflammation and Permeability Associated with the Subsequent Acquisition of Linear Growth Deficits in Infants *Am J Trop Med Hyg.* **88** 390–396
- [13] Vassileva G, Huwyler, Poirier K, Agellon LB, Toth MJ 2000 The intestinal fatty acid binding protein is not essential for dietary fat absorption in mice *FASEB J.* **14** 2040–2046
- [14] Niewold TA, Meinen M, Van der Meulen MJ 2004 Plasma intestinal fatty acid binding protein (I-FABP) concentrations increase following intestinal ischemia in pigs. *Research in Veterinary Science.* **77** (2004) 89–9

Acknowledgments

The research was funded by Directorate of Research and Community Service, Directorate General of Research and Technology Development, Ministry of Research, Technology and Higher Education in accordance with Letter of Appointment Agreement of Research Program No. 079 / SP2H / LT / DRPM / II / 2016 dated February 17, 2016.