Detection of Serum Transferrin Level in Broilers Infected with *Eimeria acervulina*

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ABSTRACT

This study was to detect the response of serum transferrin (STF) levels in broilers after infection with Eimeria acervuline. Forty broilers, one day old, were divided into 2 groups: uninfected broilers (negative control) and infected broilers with *E. acervulina* (treatment groups). At 8 days old, infected broilers were inoculated orally with 1×10^5 sporulated oocysts of *E. acervulina*. At 15 days old, serum samples were collected from all birds and analyzed by using 15% SDS-PAGE and Western Blot for the expression of STF level. The results indicated that STF was detected in both healthy and *E. acervulina*-infected broilers. The percentage of the STF concentration per the TP concentration in the treatment group was significantly ($P \leq 0.05$) higher than the negative control group. The finding of this study, STF is not a specific biomarker for *E. acervulina* infection. The monitoring of STF levels in serum should be analyzed and compared to the different health statuses of the birds. The detection of STF levels might be one of the parameters that can be used in combination with the other diagnostic techniques for predicting the gut health system in broilers.

Keywords: biomarkers, serum transferrin, broilers, *Eimeria acervulina*

INTRODUCTION

Due to the emerging antimicrobial resistance (AMR) problem in humans and poultry, reducing antibiotic use has been a concern, and the use of antibiotics as growth promoters has been banned in poultry since 2006 (European Commission, 2005; Allen, 2014; Wongyunoi, 2018). Therefore, using alternative products and gut health monitoring programs in broiler production become helpful tools for controlling infectious diseases in poultry (Dhama *et al*., 2011; Tsiouris, 2016). The gut system plays an essential role in keeping a healthy body by absorbing the digested nutrients, and it has a significant impact on broiler performance (Collett, 2012; Svihus, 2014; Kallam and Sejian, 2021). Disrupted gut integrity or gut microbiome imbalances associated with poor diet quality, stress factors, or infectious diseases can negatively affect the growth performance of broilers (Gabriel *et al*., 2006; Bailey, 2019; Bindari and Gerber, 2022).

Coccidiosis is an important protozoan disease that causes huge economic losses in the poultry industry (Chapman, 2014; Chapman *et al*., 2016; Gómez-Osorio *et al*., 2021). Coccidiosis is originated from the *Eimeria* species that cause intestinal mucosal damage and imbalance of the gut microbiome in the gastrointestinal tract (GIT), resulting in reduced feed intake, poor nutrition absorption, increased mobility, and mortality rates and low productive performances (Gómez-Osorio *et al*., 2021). Currently, seven species have been reported in poultry (Williams, *et. al*., 2009; López-Osorio *et al*., 2020). However, the outbreaks of *E. acervulina, E. maxima, and E. tenella* were the most prevalent and significantly impacted broiler production in Thailand (Vereecken *et al*., 2021).

Two common techniques for the diagnosis of poultry coccidiosis are gross lesion examination and microscopic examination (Johnson and Reid, 1970). The main advantages of these methods are their low cost and ease of implementation. However, there are laborious processes, and it might be difficult to diagnose a coccidiosis lesion in a subclinical infection. Because of the limitations of traditional methods, the detection of acute phase proteins in serum has been developed and applied to monitor *Eimeria* infections in poultry (Rath *et al*., 2009; Cahyaningsih *et al*., 2018).

Several acute phase proteins such as C-reactive protein, serum amyloid A (SAA), serum transferrin (STF), alpha-1-acid glycoprotein haptoglobin, and albumin have been demonstrated to be biomarkers for inflammatory responses by infectious diseases (Petersen *et al*., 2004; Cray *et al*., 2009; Rath *et al*., 2009; Eckersall & Bell, 2010; O'Reilly, 2016). STF is one of the major acute-phase proteins and is synthesized by the liver. The function of STF is to store and transport iron.

Monitoring of STF levels can indicate inflammation and disease infection in chickens (Aguilera *et al*., 2003; Rath *et al*., 2009; O'Reilly, 2016). Several studies have been reported on the correlation between the concentration levels of STF in serum after bacterial infections such as *Gallibacterium anatis* or *Staphylococcus aureus* (Chamanza *et al*., 1999; Roy *et al.,* 2014). However, the research on the link between changing STF levels and intestinal inflammation or damage due to Eimeria infection is still limited in poultry (Rath *et al*., 2009). Therefore, the objective of this study was to detect the response of STF levels in broilers after infection with *E. acervulina*. STF is expected to be a useful indicator for monitoring gut health in poultry production.

MATERIALS AND METHODS

Animals and management

A total of 40 one-day-old Arbor Acres broilers were used in this study. All birds were raised from 1 to 15 days of age in cage pens covered by mosquito nets, and they were reared in groups of 20 birds per cage. All birds were given coccidiostat-free and antimicrobial drug-free feed and water ad libitum. All birds were monitored twice daily for health status, clinical signs, and welfare. Our experiment was approved by the Kasetsart University Institutional Animal Care and Use Committee (ACKU65-VET-018).

Experimental design and *E.acervulina* challenge

All one-day-old chicks were randomly assigned to two groups of 20 broilers each. Group 1 was uninfected with *E. acervulina* (the negative control), and group 2 was inoculated orally with $1x10^5$ sporulated oocysts of *E. acervulina* (the treatment group) at eight days of age. Both groups of broilers collected blood samples at 15 days of age (7 days post-inoculation) and were necropsied to assess coccidial lesions and gut health.

A strain of *E. acervulina* originated from the duodenal content of broilers infected with coccidiosis and was maintained in our laboratory at the Department of Farm Resources and Production Medicine, Faculty of Veterinary Medicine, Kasetsart University, Kamphaeng Saen campus, Thailand. The oocysts were added to a 2.5% potassium dichromate ($K_2Cr_2O_7$) solution and then incubated at 30 °C for 72 hours for sporulation. Sporulated oocysts of *E. acervulina* were immediately washed and diluted with sterile distilled water to desired concentrations before inoculation.

Blood samples were collected from all broilers (uninfected and infected groups) at 15 days of age or 7 days post-inoculation (DPI). For serum preparation, blood samples were allowed to clot and separated by centrifuging at 9,500 rpm for 5 minutes, and then serum samples were kept at -20 °C until used.

Evaluation of coccidiosis and intestinal dysbacteriosis using a macroscopic scoring system

The gross lesion score of *E. acervulina* was assessed by the method of Johnson and Reid (1970), briefly, all birds were necropsied, and the duodenal part was observed with the severity of the lesion scales from 0 – 4. Score (0): no lesion and normal intestine, (1): scattered, white plaque-like are limited to the duodenum part at either the serosal or mucosal surfaces with up to 5 lesions per square centimeter, (2): the lesions are much closer together, but not coalescent and the intestinal wall show no thickening, and the content is normal, (3): the lesions are completely coalescent, the intestinal wall is very thickening, and the content is watery, and (4): the lesions are completely coalescent and appear till to the jejunal part, the intestinal wall is very thickening, and the content is filled with creamy exudate. While the lesion score of dysbacteriosis was performed according to the criteria in previous studies (Teirlynck *et al*., 2011; Bootsri *et al*., 2017). There were six parameters including (1) dilatation, (2) mucosal tone, (3) intestinal wall, (4) mucosal lesions, (5) abnormal contents, and (6) feed particles.

Identification of serum transferrin and densitometric calculation

Twenty serum samples from both groups (not shown) were analyzed using 15% SDS-PAGE under non-reducing conditions. Serum was diluted at a ratio of 1:30 with a non-reducing sample loading buffer and boiled for 5 minutes. 10 µL of each sample were loaded into gels and electrophoresed at 80 volts for 120 minutes until complete separation was achieved. After electrophoresing gels, the electrophoresed gels were then scanned using a Bio-Rad GS-800 calibrated densitometer. The formula of area × optical density was used to calculate the densities of major protein bands, and the total serum proteins of each sample (based on the mean optical density per square millimeter (mm^2)) were used to adjust the densities of major protein bands. Bovine serum albumin (BSA) was used as the standard protein concentrations of 200 ng, 400 ng, 600 ng, and 800 ng. Two serum samples from the negative control and treatment groups were shown in the density band (Figure 1).

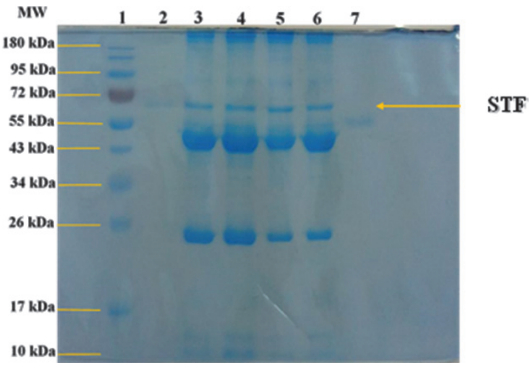

Figure 1 15% SDS-PAGE protein profiles of STF. From left to right, Lane 1: PageRuler Prestained Protein Ladder (#26618); Lane 2: chicken transferrin (400 ng); Lane 3 and Lane 4: serum sample originated from the negative control group; Lane 5 and Lane 6: Treatment group (*E. acervulina* infection); Lane 7: BSA (400 ng); MW = molecular weight.

Identification of serum transferrin by western blot analysis

To identify serum transferrin, 15% SDS-PAGE under non-reducing conditions was used with the same method as described above. Two serum samples from each group were used to confirm the STF protein band in figure 2. Therefore, the proteins in the gel were transferred onto a nitrocellulose membrane using the Bio-Rad Blot SD semi-dry transfer machine. The protein transfer process was accomplished at a constant voltage of 250 volts for 20 minutes. Non-specific proteins were blocked by soaking the nitrocellulose membrane in 5 % nonfat dry milk (w/v) in tris-buffered saline with 0.05 % Tween 20 (TBST) at 4 °C overnight. Rabbit anti-chicken transferrin antibodies used as the primary antibodies (Accurate Chemical & Scientific Corporation, AIA8240, AXELL®, USA) were added to the nitrocellulose membrane (1:5000) and incubated at 37 ºC for 1 hour. They were then washed three times with TBST solution, each time for at least 5 minutes.

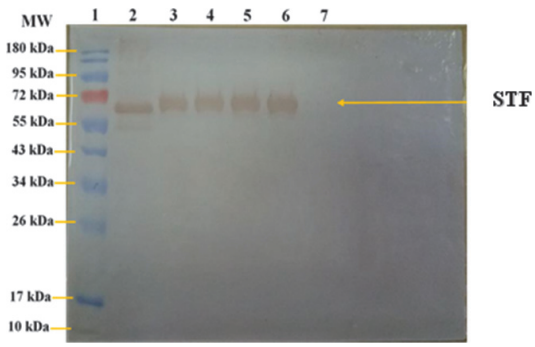

Figure 2 Western blot protein profiles of serum samples originated from the negative control group and from the *E. acervulina* infected group. From left to right, Lane 1: PageRuler Prestained Protein Ladder (#26618); Lane 2: chicken transferrin (400 ng); Lane 3 and Lane 4: serum sample originated from the negative control group; Lane 5 and Lane 6: serum sample originated from the chicken infected with *E. acervulina*; Lane 7: BSA (400 ng); MW = molecular weight.

Peroxidase-conjugated goat anti-rabbit IgG (H+L) used as the secondary antibodies were added to the nitrocellulose membrane (1:10,000) and incubated at 37ºc for 1 hour and then were washed three times with TBST solution at least 5 minutes for each time. Finally, 3, 3'-diaminobenzidine (DAB)-peroxidase substrate solution (0.05% DAB, 0.015% H_2O_2, 0.01M PBS, pH 7.2) was added to the membrane until the protein bands developed. The chicken serum transferrin was used as a positive control for the commercial products (Accurate Chemical & Scientific Corporation, AIA8240, AXELL®, USA).

Determination of total serum protein and serum transferrin

The total serum protein (TP) concentration was analyzed by using the Bradford method (He. 2011) in all serum samples (Thermo Scientific PierceTM BCA Protein Assay Kit, USA). The serum samples were diluted at a ratio of 1:30 in 9 percent normal saline. BSA was used as a standard for protein concentration. The duplicate serum samples and BSA were tested in compliance with the manufacturer. 25 µL of BSA and serum samples were transferred into each microplate well, and 200 µL of the working reagent (provided by the manufacturer) was added to each well. The plates were then thoroughly mixed on the plate shaker for 30 minutes before being incubated at 37 °C for 30 minutes. The concentration of TP in each sample was analyzed by using an Infinite F50 robotic absorbance plate reader (TECAN, Switzerland) at a 595 nm wavelength range. The percentage of STF per the total serum proteins was calculated by this formula:

$$\% \text{ Serum STF /TP} = \frac{\text{The concentration of serum STF (mg/ml)}}{\text{TP (mg/ml)}} \times 100$$

Statistical analysis

Independent samples t-test (R Studio) of inferential statistics was used to analyze and compare the coccidiosis lesion score, dysbacteriosis lesion score, STF protein concentration, TP concentration, and percentage of STF protein concentration per TP concentration between uninfected and infected groups at the same time point, and a statistically significant difference was interpreted as $P \leq 0.05$. Quantitative data were also calculated as a mean and standard deviation (SD). Categorical data analysis was used to test for differences in the distribution of individual lesion scores.

RESULTS

Evaluated coccidiosis lesion scoring and intestinal dysbacteriosis scoring

The average coccidial lesion score of the treatment group (*E. acervulina* infection) was significantly ($P \leq 0.05$) higher than the lesion score of the negative control group (Table 1). No gross intestinal dysbacteriosis lesions were observed in the negative control group except for feed particle (parameter No.6), whereas the treatment group's intestinal dysbacteriosis revealed lesions of the intestinal dilatation (3 broilers, 15%), the intestinal wall in the duodenum (14/20 broilers, 70%) and jejunum (9/20 broilers, 30%), mucosal lesions in the duodenum (17/20 broilers, 85%), jejunum (13/20 broilers, 65%), and ileum (3/20 broilers, 15%), and feed particles (18/20 broilers, 90%). Overall, the average IDL score in the *E. acervulina*-infected group was significantly ($P \leq 0.05$) higher than that in the negative control group (Table 1).

Expression analysis of serum transferrin (STF) protein

STF protein expression was determined using 15% SDS-PAGE under non-reducing conditions, followed by western blot analysis. The molecular weight of the STF protein band was from 55 to 72 kDa when compared with the positive control band. The results revealed that the expression of STF protein was identified in serum samples from both the negative control and treatment groups (Figures 1 and 2). No significant differences in the concentration level of STF protein were observed in the negative control and treatment groups ($P>0.05$), whereas the

Table 1 The intestinal dysbacteriosis lesion (IDL) scores of all broilers in each group.

Group	Intestinal dysbacteriosis evaluation						Average IDL score (Total score)	Average coccidial lesion score
	1. Dilata-tion	2. Muscle tone	3. Intestinal Wall (d/j/i/c)	4. Mucosal Lesions (d/j/i/c)	5. Total abnormal content	6. Feed particle		
Normal control (n=20)	0	0	0	0	88	16	5.2[a] (104)	0.0[a]
E. acervulina Infected (n=20)	3	0	23 (14/9/0/0)	33 (17/13/3/0)	156	18	11.65[b] (233)	2.1[b]

d = duodenum, j = jejunum, i = ileum, c = cecum, IDL = intestinal dysbacteriosis lesion

average percentage of the STF protein concentration per the TP concentration in the treatment group was significantly (P≤0.05) higher than that in the negative control group (Table 2).

DISCUSSION

Damage to the gastrointestinal tract can have an impact on broiler production performance. Acute-phase proteins of various types can be used as biomarkers in chickens to predict inflammatory processes or disease infection. The results of this study showed that the ratio between STF and TP concentrations (STF/TP) in broilers infected with *E. acervulina* was higher than the STF/TP ratio in the negative control group. In this study, the reduction of TP concentration from the broilers infected with *E. acervuline* was caused by feed intake, protein loss, low protein synthesis, and utilizing protein for repairing. The decrease in TP concentration from chickens infected with Newcastle disease could be a result of reducing in albumin content resulting from necrosis of the liver, renal tubular, and liver damage that the albumin could not synthesize the proteins in standard quantity (Kaneko, 1997; Oladele *et al*., 2005).

Our findings are in agreement with the previous studies of the expression of transferrin in chickens. They demonstrated that increasing transferrin levels in chicken serum might be induced by traumatic or infectious diseases such as *Staphylococcus* spp*., Gallibacterium anatis, infectious bronchitis* (IBV)*, and Eimeria spp*. in chickens (Chamanza *et al*., 1999; Rath *et al*., 2009; Roy *et al*., 2014). Furthermore, STF levels have been observed in healthy chickens (Xie *et al*., 2002c; Rath *et al*., 2009). The difference in ages, breeds, types of birds, or nutritional management might also affect the different levels of acute phase proteins and total protein in serum.

Therefore, STF is a non-specific acute phase protein that can be found in both healthy and diseased chickens. The monitoring of the changing STF levels in serum should be analyzed and compared to the different health

Table 2 The average concentration of STF protein and total serum protein (TP) in each group.

Group	Number of Samples	Avg. STF (mg/ml) (SD)	Avg. TP (mg/ml) (SD)	Avg. of % STF/ TP (SD)
Negative control	10	1.41 (0.29)	21.98[a] (1.69)	6.45[a] (1.45)
E. acervulina Infected	10	1.74 (0.55)	18.85[b] (3.12)	9.46[b] (3.69)

Avg = Average, SD = standard deviation, mg = milligram, ml = milliliter, n = number, TP = total protein

statuses of the birds. The detection of STF levels might become one of the diagnostic tools that can be used with the other diagnostic techniques for predicting gut health in broilers. For a better understanding of the association between STF levels and coccidiosis, further experiments are needed.

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