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Molecular and immune-histochemical changes in broiler chickens infected with *Eimeria tenella* and the protective effect of some anti-coccidial drugs

Ghada M. Fathy[1*], Khalid M. Fararh[2], Saher S. Abd El-hamied[1]

[1]*Department of Clinical Pathology, Animal Health Research Institute (AHRI), Agriculture Research Center (ARC), Zagazig, Egypt.*
[2]*Department of Clinical Pathology, Faculty of Veterinary Medicine, Benha University, Egypt.*

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***Correspondence:**

Corresponding author: Ghada M. Fathy
E-mail address: ghadafathyclinicalpathology@gmail.com

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ABSTRACT

Chicken coccidiosis is an inflammatory disease caused by the apicomplexan protozoal parasite *Eimeria*. Pro-inflammatory cytokines and chemokines activate hepatocyte receptors in the liver, altering the production and release of numerous proteins known as acute phase proteins (APPs), which eradicate pathogenic germs, repair tissue damage, and promote health. These proteins have a significant impact on defense systems. The current investigation evaluated the levels of several acute phase proteins (haptoglobin (PIT54), ceruloplasmin, and fibrinogen) in the blood, their gene expression in the liver as well as the immunohistochemistry changes in the caecum and bursa of Fabricius of chickens infected with *E. tenella* before and after treatment with two different anticoccidial drugs (diclazuril and toltrazuril). The obtained results revealed that *E. tenella* infection in chickens produced considerable activation of numerous APPs, including haptoglobin, fibrinogen, and ceruloplasmin. Furthermore, the challenged birds' caecum showed a high sensitivity to CD4, CD8, and SIgA. Anticoccidial medicines such as diclazuril or toltrazuril could considerably lower such APPs indicators and immunohistochemical alterations. In conclusion, haptoglobin, fibrinogen, and ceruloplasmin are considered as biomarkers for the APPs measurement in birds challenged with *Eimeria*. Moreover, diclazuril and toltrazuril are regarded as ideal anticoccidial medications to be used during chicken farming.

Introduction

The poultry industry is rapidly expanding and considered as an important sector in the food economy, and it is projected to continue to rise in importance. This development is the result of the chicken's exceptionally high feed conversion ratio, which makes it a profitable and sustainable meat source (Darwish *et al*., 2018). According to Chamanza *et al*. (1999) and Cray *et al*. (2009), local inflammation in poultry is accompanied by a systemic reaction known as the acute phase response (APR). The innate immune system's early, generalized response to systemic or local perturbations is known as the APR (Cray *et al*., 2009).

According to O'reilly and Eckersall (2014) and Sevimli *et al*. (2015) cytokines, which operate as cell growth factors and as pro- or anti-inflammatory mediators of inflammation, predominantly control this response. Immune cells that have been activated produce and release these cytokines. Pro-inflammatory cytokines and chemokines stimulate the liver's hepatocyte receptors, changing the production and release of several proteins known as acute phase proteins (APPs) that eliminate pathogenic microorganisms, repair tissue damage, and promote health. According to Murata *et al*. (2004), these proteins have a major impact on defensive systems.

C-reactive protein (CRP), haptoglobin (Hp), ceruloplasmin (Cp), serum amyloid A (SAA), alpha-1-acid glycoprotein (AGP), and fibrinogen are examples of positive APPs found in birds, while albumin and transferrin (TRF) are examples of negative APPs (Chamanza *et al*., 1999). Adler *et al*. (2001) found that the concentration of APPs rises as inflammation progresses in birds. In order to monitor the health of the birds and gauge the severity of inflammation, as well as for clinical diagnostics, the blood content of APPs in poultry is frequently measured (Xie *et al*., 2002; Seifi *et al*., 2014; Rath *et al*., 2015).

Chicken coccidiosis is an inflammatory illness brought on by an apicomplexan protozoal parasite of the genus *Eimeria* (Chapman, 2014). According to Bussière *et al*. (2018), these coccidian organisms are common intestinal protozoans in chicken and are known to induce acute intestinal problems. There are seven different species of *Eimeria*, but according to Thenmozhi *et al*. (2014), *E. tenella*, *E. maxima*, and *E. acervulina* are the most economically relevant ones. According to Zaman *et al*. (2012), *E. tenella* is the most common coccidia found in poultry. It lives in the caeca and causes a severe illness that is marked by intestinal bleeding, high morbidity, high mortality, a reduction in weight gain with emaciation, a loss of skin pigmentation, and bloody cores that are present with clusters of sizable schizonts and oocysts in the caecum (Habibi *et al*., 2016).

Knowing the levels of APPs in poultry allows for early detection of inflammatory processes and forms the basis for taking medical action to reestablish homeostasis (Hochepied *et al*., 2003).

This study aimed to measure the levels of several acute phase proteins (haptoglobin (PIT54), ceruloplasmin, and fibrinogen) in the blood, their gene expression in the liver and the immunohistochemistry changes in the caecum and bursa of Fabricius of chickens infected with *E. tenella* before and after treatment with two different anticoccidial drugs (diclazuril and toltrazuril).

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Materials and methods

Birds

At one day old, 100 Cobb mixed breed broiler chicks were bought from Al-Kahira Poultry Company. The chicks were divided into four groups at random. Group 1 was left as a control negative. Group 2 was infected with a 1 ml solution containing roughly 100000 sporulated *E. tenella* oocysts using the intra-crop route at 14 days of age, and Group 3 was infected with the same dose of sporulated *E. tenella* oocysts on the 14th day old chicks and then treated with diclazuril 1 ml/4 liter drinking water at the fifth day P.I. (post infection) . Group 4 was given the same quantity of sporulated *E. tenella* oocysts and subsequently, on the fifth day P.I., got toltrazuril 7 mg/kg b.wt.

Diclazuril

Pharma Swede Company in Egypt provided the Diclazuril Diclosol® suspension, 10 mg/ml. It was supplied in drinking water at a concentration of 2.5 ppm (1 ml/4 liters of water).

Toltrazuril

A therapeutic dose of 7 mg/kg body weight of toltrazuril solution (Toltrasol 2.5% ARABCOMED Company) was given.

Sampling

Five birds from each group were used. At the age of 21 and 28 days, two blood samples were collected. The first blood sample was taken from the wing vein into tubes without anticoagulant. The sera were separated by centrifugation at 4000 g for 15 minutes and kept at -20°C until use. Acute phase proteins (haptoglobin and ceruloplasmin) were assessed in the serum of various groups of birds using approved standard techniques. The second sample was collected on sodium citrate to separate plasma for fibrinogen level determination. Birds were slaughtered, and tissue samples were collected from the liver for total RNA isolation, and from the caecum and bursa of Fabricius for immunohistochemistry examination.

Laboratory examination

To determine the levels of acute phase proteins in the serum, ELISA kits specific for chicken Hp and CP (Wuhan Fine Biotech Co., Ltd., East Lake High-tech Development District, Wuhan, Hubei Province, China) were used. Plasma fibrinogen concentration was performed according to Becker *et al.* (1984).

Real-Time PCR analysis

Total RNA was extracted from the tissue samples using QIAamp RNeasy Mini kit (Qiagen, Germany, GmbH). In brief, 200 µL of the extract were added to 600 µL RLT buffer containing 10 µL β-mercaptoethanol per 1 mL, incubated at room temperature for 10 min. One volume of 70% ethanol was added to the cleared lysate, and the steps were completed according to the purification protocol of the QIAamp RNeasy Mini kit (Qiagen, Germany, GmbH). Primers used for PCR amplification were supplied from Metabion (Germany) and listed in Table 1. Primers were utilized in a 25- µL reaction containing 10 µL of the 2x HERA SYBR® Green RT-qPCR Master Mix (Willowfort, UK), 1 µL of RT Enzyme Mix (20X), 0.5 µL of each primer of 20 pmol concentration, 3 µL of water, and 5 µL of RNA template. The reaction was performed in a step one real time PCR machine in Biotechnology unit, Animal Health Research Institute, Zagazig. Amplification curves and CT values were determined by the step one software. To estimate the variation of gene expression on the RNA of the different samples, the CT of each sample was compared with that of the positive control group according to the "ΔΔCT" method stated by Yuan *et al.* (2006).

Immunohistochemistry analysis

In the bursa and caecum of infected and control hens, we labeled CD4 and CD8 T cells using the direct peroxidase immunohistochemical technique. Rabbit antibodies produced against human T-CD4 and T-CD8 cells were utilized in commercial kits (DAKO EPOS, N0U 0026). 3-5 m thick tissue samples were fixed in 99.9% methanol for 48 hours, and then treated in xylol twice for 15 minutes. The tissue samples were then rehydrated in a series of alcohol dilutions (100%, 96%, 70%, and 50%), and then rinsed in distilled water. Following the application of anti-CD3 antibodies (Anti CD3/HRP) for 60 min at room temperature, the samples were three times washed in TBS (Tris Buffered Saline, pH=7.5) after being blocked for 5 min by endogenous peroxidase in 3% H_2O_2. The next step was three TBS rinses, each lasting five minutes. The same procedure was used to treat the control samples using only TBS. Hematoxylin was employed for contrast staining after the reaction was visualized with 0.05% DAB-H_2O_2 in 0.1 M imidazole-HCl (pH=7.1) for 10 min. The samples were then processed for microscopic examination and coated in Canada balsam.

Statistical analysis

The data were presented as mean±standard error. One-way analysis of variance (ANOVA), F-test was used to statistically compare mean values between the experimental groups using the SPSS 16 software. According to Norusis (2008), a P value of 0.05 or lower was deemed statistically significant. For PCR and correlation analysis, data were edited in Microsoft

Table 1. Primers sequences, target genes, amplicon sizes and cycling conditions for SYBR green RT-PCR.

Target gene	Primers sequences	Reverse transcription	Primary denaturation	Amplification (40 cycles)			Reference
				Secondary denaturation	Annealing (Optics on)	Extension	
B- actin	CAACACAGTGCTGTCTGGTGG ATCGTACTCCTGCTTGCTGAT						Abdul-Careem *et al*. (2008)
Fibrinogen	TCAGGCAGAGAATGTGAGGA GGCTTGGTAAAAGGATCTGGC						Tomas Marques (2017)
PIT 54 (Haptoglobin-like protein)	GCCAGTGCAATTTGTTCAGA TCCCGTAAATCCCAGTTGTC	50˚C 30 min.	94˚C 15 min.	94˚C 15 sec.	55˚C 30 sec.	72˚C 30 sec.	Tomas Marques (2017)
Ovotransfenrin	CACTGCCACTGGGCTCTGT GCAATGGCAATAAACCTCCAA						Niu *et al*. (2018)
Ceruloplasmin	GAGAGTAAGGGTGGGGGTGGG TATTTCACATTTTCCACAAGG						Bello *et al*. (2016)

Excel (Microsoft Corporation). The Levene and Shapiro–Wilk tests were used in order to check the normality and homogeneity of variance (Razali and Wah, 2011). Two way ANOVA according to the statistical analysis system (Proc ANOVA; SAS, 2012) was used for assessing different gene expression. The mathematical model was $Y_{ijk} = \mu + TRT_i + Time_j + TRT*Time_k + e_{ijk}$ where Y is the studied gene; μ is the overall mean, TRT_i is the effect of treatment and e_{ijk} is the error. In case of being significant effects of the treatment, multiple comparisons among means were carried out with Duncan's multiple range test (Steel *et al*., 1980). Statistical significance was accepted as $p < 0.05$. Results were expressed as means±SE

Results and Discussion

Acute phase proteins (APPs) are blood proteins that are largely produced by the liver as part of a complicated systemic response known as the acute phase response (Petersen *et al*., 2004; Ceron *et al*., 2005). The quantification of APPs in veterinary medicine has been proposed to have diagnostic and prognostic utility in the study of disease and infection. Their profiles are utilized diagnostically and for understanding the pathophysiology of significant disorders (Petersen *et al*., 2004; Eckersall and Bell, 2010). *Eimeria* species enter the mucosa and destroy the epithelial layer of infected intestinal cells, causing necrosis and inflammation (Cheng *et al*., 2018).

In the current investigation, blood levels of HP (PIT 54), ceruloplasmin, and fibrinogen were assessed as biomarkers for assessing the inflammatory processes associated with *E. tenella* infection in chickens.

The obtained results revealed an activation of such biomarkers upon infection with *E. tenella*. For instances, at 21 days of age, PIT 54 was activated from 261.2±2.2 (Control group) to 313.4±1.8 (diseased group). Likely, ceruloplasmin was activated from 40.6±1.6 (Control group) to 109.8±1.8 (diseased group). Besides, fibrinogen was activated from 282.3±1.6 (Control group) to 426.5±1.4 (diseased group). Similar phenomenon was also recorded when such parameters were re-measured at 28 days of age. Interestingly, treatment with diclazuril (G3), and toltrazuril (G4) significantly reduced such activated biomarkers, particularly when treated with dicazuril indicating the recovery of the birds from the state of inflammation produced by *E. tenella* (Table 2).

Herein, there were significant effects of different treatments on the transcription of all considered genes (p=0.0001). Meanwhile, time points and their interaction with treatment had no significant effects (p>0.05; Table 3). Generally, the positive control was significantly higher than the negative control and the other treated groups (p<0.05 Fig. 1B-D) . Non-significant differences were observed between the diclazuril and toltrazuril-treated groups for all studied genes. Correlation analysis between mRNA expression and protein activity of the tested markers indicated positive correlations, particularly in the case of PIT54, and CB as shown in Fig. 2 indicating activation of such markers in a mechanistic way.

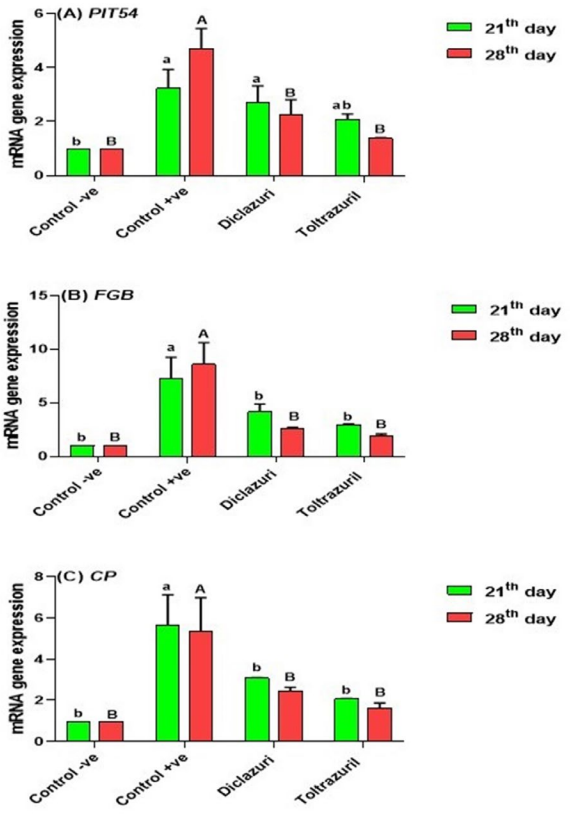

Fig. 1. Treatment by time interaction (means±SEM) effects on the transcription of PIT54, FGB, and CP; [a, b, c] Means within a row without a common superscript letter differ at p < 0.05 at 10 days; [A,B,C] Means within a row without a common superscript letter differ at p < 0.05 at 17 days.

Haptoglobin is an alpha-2 globulin that is known as PIT 54 in chickens. Haptoglobin's major role is to bind free hemoglobin produced from erythrocytes and decrease its oxidative activity (Yang *et al*., 2003). It also binds hemoglobin to reduce iron losses via urine after hemolysis, hence

Table 2. Effect of *E. tenella* on some biochemical parameters (ceruloplasmin, fibrinogen, and haptoglobin (PIT 54) in different experimental groups.

	G1	G2	G3	G4
10 days post-infection				
Ceruloplasmin (mg/L)	40.6±1.6^{c}	109.8±1.8^{a}	51.9±1.5^{b}	54.0±1.0^{b}
Fibrinogen (g/L)	282.3±1.6^{d}	426.5±1.4^{a}	310.9±1.4^{c}	323.6±1.8^{b}
Haptoglobulin (PIT 54) (g/L)	261.2±2.2^{d}	313.4±1.8^{a}	285.7±2.2^{c}	295.2±1.0^{b}
17 days post-infection				
Ceruloplasmin (mg/L)	44.9±1.6^{c}	134.4±1.2^{a}	72.2±1.2^{b}	77.7±1.7^{b}
Fibrinogen (g/L)	292.3±1.7^{d}	418.3±1.5^{a}	297.3±1.8^{c}	306.7±6.8^{b}
Haptoglobulin (PIT 54) (g/L)	223.6±2.1^{d}	374.3±1.3^{a}	232.3±1.1^{c}	253.7±1.02^{b}

[a, b, c] Means within a row without a common superscript letter differ at p < 0.05

Table 3. Effect of treatment, sampling time and their interaction on the transcription of PIT54, FGB, OV, and CP.

Items	Treatment (T)				*p* -Value		
	Control +ve	TA	TD	Control -ve	T	Time (Ti)	T × Ti
PIT54	3.97±0.80^{a}	2.47±0.55^{b}	1.72±0.26^{bc}	1.00±0.00^{c}	<0.0001	0.82	0.14
FGB	7.94±1.82^{a}	3.36±0.68^{b}	2.41±0.37^{bc}	1.00±0.00^{c}	<0.0001	0.65	0.53
CP	5.53±1.37^{a}	2.79±0.22^{b}	1.86±0.21^{bc}	1.00±0.00^{c}	0.00	0.54	0.98

[a, b, c] Means within a row without a common superscript letter differ at p < 0.05

sparing tissues from harm caused by free hemoglobin (Petersen *et al*., 2004). Haptoglobin has been identified as a potent angiogenic agent essential for endothelial cell proliferation and differentiation in the development of new blood vessels. As for the results of haptoglobin, the experimental challenge with *E. tenella* caused an increase in the level of PIT 54 from the tenth day post inoculation till the end of the experiment and that corresponds to what has been previously reported by Georgieva *et al*. (2010), also it was stated by Freitas *et al*. (2011) and Petrova *et al*. (2022) who assured that rabbits infected with E. stiedae had increased level of haptoglobin.

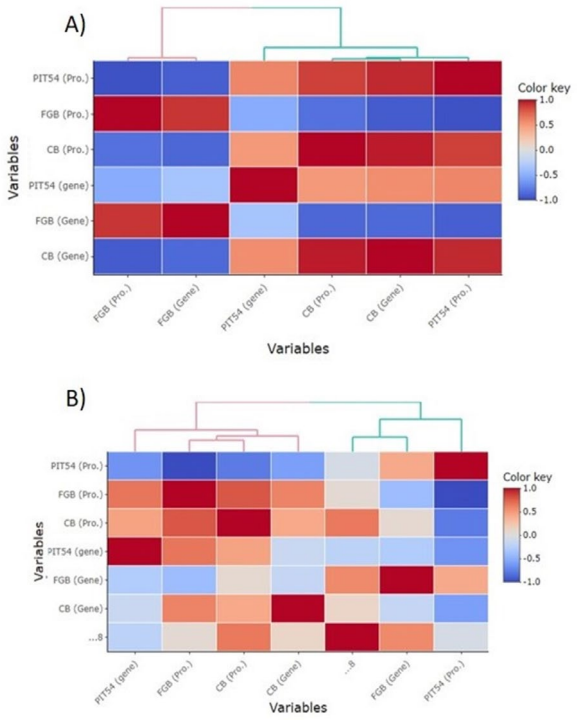

Fig. 2. Correlation analysis between the protein and mRNA expressions of the examined FGB, CB, and PIT 54 at A) 10 days post infection, B) 17 days post infection.

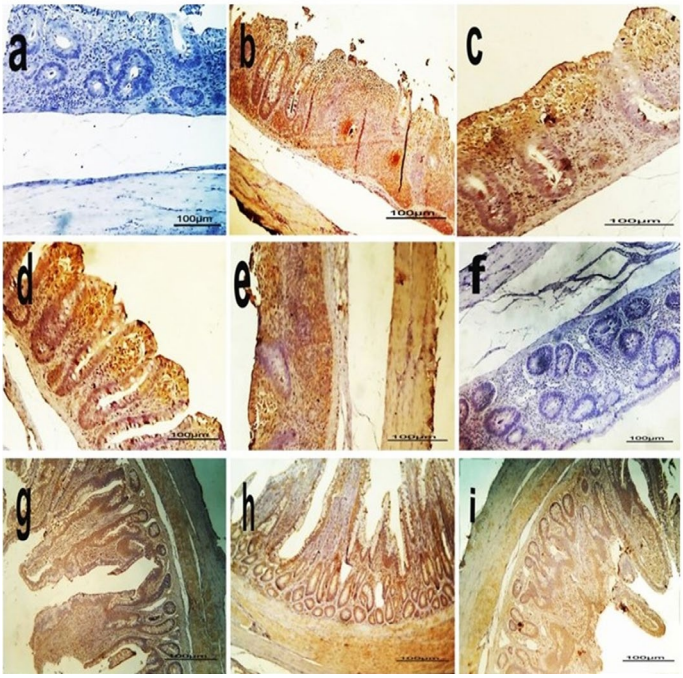

Fig. 3. Photomicrographs of peroxidase stained sections of chicken's caecum at 28th day showing a). G. 1 (control group) with complete negative expression for CD8. b, c, d) G. 2 (infected group) with moderate to severe positive expression for CD4, CD8 and secretory IgA consequently. e, f) G. 3 (infected and diclazuril treated group) with moderate positive and negative expression for CD4 and CD8 consequently. g, h, i) G. 4 (infected and toltrazuril treated group) with moderate to mild positive expression for CD4, CD8, and secretory IgA consequently. Scale bar: = 100 µm.

Johnson *et al*. (2009) identified fibrinogen (FB) as the first APR protein. It is broken down by thrombin in the coagulation cascade to form fibrin (Harr *et al*., 2015). It is a soluble glycoprotein found in the plasma of all vertebrates and is produced by hepatocytes (Ceron *et al*., 2005). FB is an important regulator of inflammation during illness. The ability of FB, fibrin, and derived peptides to bind to and activate a wide spectrum of immune cells is connected with their pro-inflammatory effects. During an APR, the vascular disruption caused by pathological processes such as inflammation, infection, and tissue injury causes an increase in fibrinogen concentration in the blood (Davalos and Akassoglou, 2012). FB also serves as a matrix for inflammatory-related cell migration and tissue healing (Murata *et al*., 2004). The results of our investigation demonstrated a significant increase in plasma fibrinogen levels in the *E. tenella*-infected group (G2). Our findings agreed with those of Gonkowska *et al*. (2004) and Georgieva *et al*. (2010), who found that co-infection with *E. coli* and *E. tenella*, as well as a single infection with either *E. coli* or *E. tenella*, raised plasma fibrinogen levels. Koĭnarski and Nikolov (1987) discovered an increase in fibrinogen levels in turkeys infected with *Eimeria* adenoeides.

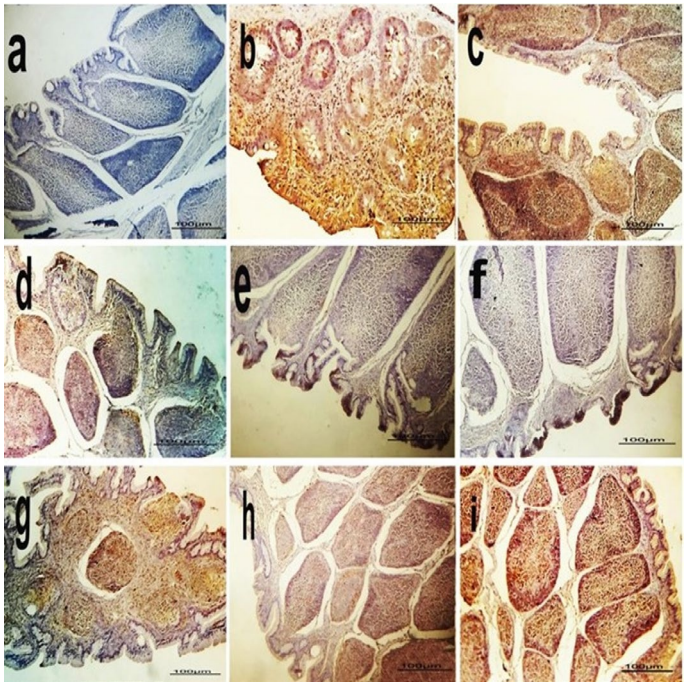

Fig. 4. photomicrographs of peroxidase stained sections of chickens' bursa at 28th day showing a). G. 1 (control group) with complete negative expression for CD4. b, c, d) G.2 (infected group) with severe to moderate positive expression for CD4 ,CD8 and secretory IgA consequently. e, f) G. 3 (infected and diclazuril treated group) with negative expression for CD8 and secretory IgA consequently. g, h, i) G. 4 (infected and toltrazuril treated group) with moderate to mild positive expression for CD4, CD8 and secretory IgA consequently Scale bar = 100 µm.

Ceruloplasmin (CP), like other APPs, is primarily synthesized in the liver, primarily in the cytoplasm of hepatocytes. Its primary purpose is to transport and store copper within the body, and it also possesses anti-oxidative properties. In vivo investigations have revealed that CP can oxidize the Fe2+ ion, hence reducing the iron-induced oxidation effect (Floris *et al*., 2000). Our findings revealed a considerable increase in ceruloplasmin levels in the infected non-treated group (G2). The increase in circulating ceruloplasmin may be an acute phase response to the possibility of secondary bacterial infection, or it may be caused by *E. tenella* causing intestinal wall erosion and subsequent inflammation caused by an infiltration of macrophages secreting IL-1 into the damaged tissues (Augustine and Richards, 1988). This is consistent with the findings of Georgieva *et al*. (2010), who found that *Eimeria* infection dramatically elevated serum ceruloplasmin levels. Gonkowska-Mazur *et al*. (2004) also found an increase in ceruloplasmin levels in *E. coli*-infected hens.

When compared to infected and untreated group (G2), diclazuril (G3) or toltrazuril (G4) treatment reduced the levels of Hp, Cp, and FB. This could be because HP, Cp, and FB are acute phase proteins that contribute

to restoring homeostasis and inhibiting microbial growth after infection, inflammation, or stress (Murata *et al*., 2004). As a result, the two anti-coccidial treatments diclazuril and toltrazuril considerably reduced the levels of the tested APPs. This finding lends credence to the idea that employing anticoccidial medicines could minimize factors that cause systemic inflammation or infection in broiler hens. As a result, inflammation or infection is reduced even further. Those findings are consistent with Lee *et al*. (2012), who found that most anticoccidial medications reduced the level of alpha-1 acid glycoproteins as an APP in chicken coccidiosis.

The fundamental component of protective immunity against *Eimeria* is T-cell-mediated immunity by intestinal intraepithelial lymphocytes (Lillehoj and Lillehoj, 2000). Due to their ability to combat intracellular infections such as coccidia, CD4+ T helper (Th) cells and CD8+ cytotoxic T lymphocytes (CTLs) are the two key T-cell subsets implicated in anticoccidial immunity (Yun *et al*., 2000). Our immunohistochemical markers CD4, CD8, and SIgA intensity when measured on the 28th day revealed a positive intense reaction in both the caecum and bursa of Fabricius of infested chickens. Mild reactions against such markers were observed in caecum of both diclazuril and toltrazuril treated groups. The percentage area occupied by CD4+ and CD8+ cells was significantly higher in infected chickens than in non-infected chickens. The increased area filled by T helper and cytotoxic lymphocytes suggests that there are more of these cells at the site of parasite proliferation and that specialized effector cell driven immune actions against parasitic stages have begun in caecal tissue (Figs. 3 and 4). Rothwell *et al*. (1995) proposed that CD4+ and CD8+ cells have a role in providing resistance to initial and subsequent coccidial infection. Increased T cell numbers are associated with increased production of the proinflammatory cytokine interferon (IFN)-, which has an immunoregulatory effect (Burke and Young 2019), as well as limiting parasite intracellular development (Lillehoj, 1998). Likely, Vervelde *et al*. (1995) and Bessay *et al*. (1996) found an increase in the numbers of CD4+ and CD8+ cells in the ceca of *E. tenella*-infected chickens. Gadde *et al*. (2013) discovered an increase in the percentage of CD4+ and CD8+ cells in E. adenoids-infected poults.

Conclusion

E. tenella infection in chicken causes significant activation of several APPs (haptoglobin, fibrinogen, and ceruloplasmin). Moreover, a strong reaction against CD4, CD8, and SIgA occurs in the caecum and bursa of the Fabricius of the challenged birds. Treatment with the anticoccidial drugs, diclazuril or toltrazuril could significantly reduce such APP markers and immunohistochemistry changes.

Conflict of interest

The authors declare that they have no conflict of interest.

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