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HEMATOLOGICAL CHANGES ASSOCIATED WITH MIXED INFECTION OF COCCIDIOSSIS AND NECROTIC ENTERITIS IN TURKEY

DOAA I.A. MOSTAFA [1] AND SANAA M SALEM [2]

[1] Department of Clinical Pathology, Animal Health Research Institute (AHRI), Zagazig Branch, Agriculture Research Center (ARC), Egypt.

[2] Department of Pathology, Animal Health Research Institute (AHRI), Zagazig Branch, Agriculture Research Center (ARC), Egypt.

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ABSTRACT

Necrotic enteritis is a significant problem to the poultry industry all over the world. Little information exists concerning the pathogenesis, immunity or experimental induction of necrotic enteritis in turkeys. Therefore, the present work was designed to develop an experimental model of coccidiosis and necrotic enteritis in turkey for detection of the hematological changes associated with it with trial to overcome using amoxicillin and diclazuril. 40 one day old turkey poults were used for this, divided randomly into 4 equal groups. Gp. (1) left as normal control, gps. (2, 3 and 4) infected experimentally with *Eimeria* spp, *Clostridium perfringens* and mixed infection respectively and treatment of groups 2, 3, and 4 by amoxicillin, diclazuril and (amoxicillin+diclazuril) respectively by the appearance of symptoms. The study revealed anemia in gps. 2, 3 and 4, with increase in the platelets count in gps. 2 and 4 and increase in the RBCs osmotic fragility in gps. 3 and 4. In addition to, increase in the total and differential leucocytic count in all infected gps. The amoxicillin and diclazuril had significant improvement in all associated changes.

***Keywords**: Clostridium perfringens*- *Eimeria* spp- Turkey - Hematogical changes- Osmotic fragility

INTRODUCTION

Turkey is important poultry species grown in Egypt and of high economic importance, but their industry exposing many problems. Necrotic Enteritis (NE) and Coccidiosis are widespread diseases of considerable economic importance affecting the turkey industry, as it causes high losses among birds in addition to the high cost of its control. It is caused by mixed infection of both *Clostridium perfringens* and *Eimeria spp*. (Timbermont *et al.,* 2011). Avian necrotic enteritis was first described in 1961 (Parish, 1961) and since then it has been reported to occur in almost poultry-producing countries (Mcdevit *et al*., 2006).

Clostridium perfringens is a gram-positive, rod-shaped, spore-forming, anaerobic bacterium. It is commonly found as a normal inhabitant of the gastrointestinal tract of humans and animals (Fasina *et al*., 2016).

It produces at least 12 different toxins, which are associated with the occurrence of

Corresponding author: Doaa I.A. Mostafa
E-mail address: doaaibrahim706@gmail.com
Present address: Department of Clinical Pathology, Animal Health Research Institute (AHRI), Zagazig Branch, Agriculture Research Center (ARC), Egypt.

the disease. Not all the *C. perfringens* inhabiting the gut are pathogenic, only few strains are virulent and pathogenic. They produce extracellular toxin types, named; alpha (α), beta (β), epsilon (ε), and iota (ι). They produced by *C. perfringens* biotypes A, B, C, D, and E (Paiva and McElroy 2014).The α -toxin was the major toxin involved in necrotic enteritis in poultry (Timbermont *et al.*, 2009).

The disease result in outbreaks with mortality rates reached to 50% as an acute enterotoxaemia. The clinical signs are usually of rapid onset, and often represented by severe depression followed by a sudden increase in flock mortality. The disease primarily affects broiler chickens at 2–5 weeks old and turkeys at 7–12 weeks old (Osman and Elhariri, 2013). For many years, prophylactic use of antibiotic in feed has been primary practice for controlling necrotic enteritis in broiler industry, however it may develop *C. perfringens* strain resistance antibiotic which may threatened economic stability of broiler industry (Baumgartner, 2003). Amoxicillin was most effective against 81.8% of tested isolates of *C. perfringens* isolated from turkey and had significant improvement of turkey infected cases (Sanaa *et al.*, 2020).

Coccidiosis is the most common predisposing factors of NE (Paiva and McElroy 2014). It is an important disease of the turkey caused by protozoan parasites of the genus *Eimeria*. The parasites are widespread in turkey flocks and capable of causing considerable economic loss yet, compared with the chicken, little research has been undertaken to better our understanding of the disease and develop improved methods of control (Chapman 2008). Vrba and Pakandl (2014) identified multiple *Eimeria spp.* infecting the turkey as *Eimeria meleagridis, Eimeria dispersa, Eimeria gallopavonis, Eimeria meleagrimitis* and *Eimeria innocua* and they indicated that *Eimeria adenoeides* is most probably a synonym for either *E. meleagridis or E. gallopavonis*. Moreover (Chapman 2008) reported that *E. adenoeides, E. gallopavonis, and E. meleagrimitis* are considered highly pathogenic, *E. dispersa* mildly pathogenic and E. *meleagridis, E. innocua*, and *E. subrotunda* non-pathogenic. The intestinal damage caused by coccidia is an essential predisposing factor for NE resulting in over growth of *C. perfringens* and toxin production (Assis *et al.*, 2010).

Anticoccidial compounds should be highly effective against all developmental stages of *Eimeria* species, don't effect on the host immune response as well as have no residues in the tissues. In this respect, diclazuril is one of a series of benzenacetonitrile derivatives. The prophylactic anticoccidial efficacy of diclazuril in feed was studied in chickens by (Awaad *et al.*, 2003), in addition to Sanaa *et al.* (2020) who reported that diclazuril is an effective anticoccidial drug in treatment of coccidiosis and NE in turkey.

The objective of this work is to investigate the hematological changes associated with experimental infection of C. *perfringens* and *Eimeria* species in turkeys with trial to illustrate their pathogenesis and find out the efficacy of diclazuril and amoxicillin on their control.

MATERIALS AND METHODS

Experimental birds

A total number of 40 one day old apparently healthy Balady turkey poults were used for this study; dropping of birds were examined to confirm the absence of coccidia using salt flotation (Permin and Hansen 1998). They were floor reared in separate units under hygienic measures. Turkeys were supplied with drinking water and starter feed (28%protein) ad- libitum. They were divided randomly into 4 equally groups each of 10 birds.

The infective agents

***Eimeria* spp:** obtained from Animal Health Research Institute, Zagazig branch, and used by a dose 100.000 sporulated oocysts for each bird orally.

Clostridium perfringens: inoculums of 10^8 CFU**:** obtained from Animal Health Research Institute, Zagazig branch, and used by a dose 2.5ml for each bird orally.

Preparation of *Eimeria spp:* oocysts were collected from the infected birds, scraping were made from the lesions and rinsed into potassium dichromate solution (2.5%) to release the unsporulated oocysts then it stored at 4ºc. Oocysts must undergo sporulation before they are infective. The collected oocysts washed by distilled water 3-4 times and centrifuged on 3000 rpm for 10 minutes to remove the potassium dichromate. The oocysts were counted using the hemocytometer method (Reid, 1978)

Drugs

Anticoccidial drugs: Diclazuril 10mg: Diclazuril (Pharma Swede Company) dose: 1ml/2 liter drinking water for two successive days.

Antibiotics: Amoxicillin antibiotic powder: Amoxitryl (Pharma Swede Company) dose: 20mg/kg body for 3 successive days

Experimental design

40 one day old Balady turkey poults were used. Turkeys were divided randomly into 4 equal groups. Turkeys of group (1) remain as non- infected non- treated (negative control). Group (2) was infected orally at 21day old with 100.000 sporulated oocysts of *Eimeria* spp (Dalloul *et al*., 2003). Group (3) was infected orally at day 28^{th} day old with 2.5ml of 10^8 CFU *C. perfringens* inoculums (Ferdoush *et al.*, 2014). Group (4) was infected with both *Eimeria* spp (21^{th}day old) and *C. perfringens* (28^{th} day old).

Group (2, 3, 4) were treated with diclazuril, amoxicillin and (diclazuril+amoxixillin) respectively and after the appearance of symptoms.

Sampling

Blood samples: two blood samples from five poults in each group were collected after the appearance of symptoms and one week after the treatment from the wing vein under aseptic conditions using a sterilized syrings, one on EDTA anticoagulant for hematological examination and the other on heparin anticoagulant osmotic fragility test.

Hematological studies:

Erythrocyte counts were recorded using an improved Neubauer hemocytometer and Natt and Herrick solution as a special diluent for chicken blood as described by Harrison and Harrison (1986). The packed cell volume was estimated using micro hematocrit centrifuge as described by Coles (1986) Hemoglobin level was estimated using the cyanomethemoglobin colorimetric method after centrifugation according to Van Kampen and Zijlstra (1983). Mean corpuscular volume (MCV) and mean corpuscular hemoglobin concentration (MCHC) were calculated. Blood films were prepared, fixed using methyl alcohol, and stained by Giemsa for estimating the differential leukocyte count as described by Feldman *et al.* (2000).

Osmotic fragility test: was performed using Weitherman tube, NaCl 1% and distilled water by preparation of 12 tubes of hypotonic solution of saline started at 0.8 saline solution till 0.25 saline solution, then put one drop of heparinized blood to each tube and mixing it by inverting the tubes. Leave them for 20 min. at room temperature then centrifugation at 1500 rpm for 15 min. then reading it against white background to detect the initial lysis (the first tube were the saline changed to red color) and the complete lysis (the last tube still have RBCc settled at the bottom) Miale (1982).

Statistical analysis

The data in this study were statistically analyzed by one way anova (Tamhane and Dunlop 2000) using the MSTAT-C computer program. Results are presented as mean ± SE, and the statistical significance was set at ($P \leq 0.05$). The significance between groups represented by small letters and the highest value represented by (a) letter.

RESULTS

Clinical signs, postmortem lesions:

The clinical signs observed in infected groups (2, 3 and 4) were depression, ruffled feather, bloody diarrhea and growth retardation. With regard to morbidities and mortalities, the results showed that group (4) (mixed infection with coccidia and clostridium) had higher morbidity rate (50%) and mortality higher (50%) of birds than group (2) (challenged with coccidia) (50%) morbidity and (40%) mortality of birds, finally group (3) (challenged with *clostridium perfringens* only) (40%) morbidity and (50%) mortality. One week after treatment most of these symptoms in tested groups disappeared and no mortalities were observed.

Hematological results

Compared with normal control group (1), at the appearance of symptoms, there was a significant decrease ($P \leq 0.05$) in RBCs count, Hemoglobin content, and hematocrit with significant increase ($P \leq 0.05$) in the mean corpuscular volume in gp. (2, 3 and 4). Significant decrease in mean corpuscular hemoglobin concentration (MCHC) in groups (2 and 4) which revealed macrocytic hypochromic anemia in these groups and nonsignificant changes in MCHC in group (3) which revealed macrocytic normochromic anemia (Table 1). One week after treatment with amoxicillin and diclazuril, birds in groups (2, 3 and 4), showed nonsignificant changes in RBCs count, Hemoglobin content, and hematocrit value when compared with normal control group (Table 2). The platelets count showed significant increase in gp. (2 and 4) challenged with coccidia and ciccidia and chlostridia, with nonsignificant change in gp. (3) infected with cholestridia only (Table 1). After treatment the platelets count returned to normal in all groups (Table 2).

Leukogram results

The leukogram showed a significant increase in total leukocytic count and heterophil count in groups (3 and 4) with significant increase in the eosinophilic count in groups. (2 and 4) compared with normal control group. Group (4) only showed significant decrease in basophilic count. Nonsignificant changes were seen in lymphocytic and monocytic counts in all groups (Table 1). Nonsignificant changes in all examined leukogram parameters were seen in groups (2, 3 and 4) comparing to normal control gp. one week after treatment with amoxicillin, diclazuril and their combination respectively (Table 2).

Table 1: Hematological changes of the control and tested groups (Mean±SE) (n=5).

Groups parametres	Control (1)	Coccidia (2)	Clost (3)	Clost+coccidia (4)
RBCs count (×10^{6}cells /µl)	3.01±.098^a	2.21±0.09^d	2.51±0.087^{bc}	2.44±0.01^{bcd}
Hemoglobin (gm%)	12.27±0.07^a	10.47±0.29^{bc}	11.00±0.20^{bcd}	10.4±0.2^d
PCV %	31.79±0.1^a	28.87±0.26^b	29.87±1.14^b	28.72±0.16^b
MCV fl	105.66±3.78^c	130.63±0.94^a	119.05±0.83^b	117.7±0.21^b
MCHC %	38.59±0.29^a	36.27±0.89^b	36.82±0.78^{ab}	36.23±0.88^b
Platlets count (×10^{3}cells /µl)	28.67±1.67^b	44.67±2.9^a	33.33±1.33^b	46.67±2.91^a
WBCs count (×10^{3}cells /µl)	22.43±0.03^b	22.8±0.72^b	25.75±0.38^a	26.03±0.47^a
Lymphocyte count (×10^{3}cells /µl)	12.19±0.5^b	13.31±0.36^{ab}	13.41±0.21^{ab}	13.37±0.49^{ab}
Monocyte count (×10^{3}cells /µl)	1.75±0.72	1.3±0.12	2.02±0.22	1.53±0.38
Heterophile count (×10^{3}cells /µl)	6.95±0.12^b	5.22±1.7^b	8.92±0.1^a	9.00±0.09^a
Eosinophile count (×10^{3}cells /µl)	0.37±0.08^b	0.79±0.03^a	0.44±0.09^b	0.81±0.07^a
Basophile count (×10^{3}cells /µl)	1.07±0.12^a	1.04±0.1^{ab}	0.97±0.07^{ab}	0.84±0.11^b

n: number of samples **RBCs**: Red blood cell count **PCV**: Packed cell volume
TLC: Total leukocytic count **MCV:** Mean corpuscular volume.
MCHC: Mean corpuscular hemoglobin concentration **WBCs**: White blood cell count
Significant at P≤0.05

Table 2: Hematological changes of the control and tested groups (Mean±SE) (n=5) After treatment.

Groups Parameters	Control (1)	Coccidia (2)	Clost (3)	Clost+cocidia (4)
RBCs count (×10^{6}cells /µl)	3.01±.098	2.83±0.13	2.99±0.04	2.89±0.047
Hemoglobin (gm%)	12.27±0.07	11.87±0.43	11.33±0.35	11.67±0.27
PCV %	31.79±0.1	30.59±0.61	30.58±0.44	30.15±0.49
MCV fl	105.66±3.78	108.1±4.3	102.27±0.29	104.26±3.37
MCHC %	38.59±0.29	38.79±2.26	37.05±0.74	38.68±0.47
Platlets count (×10^{3}cells /µl)	30.02±2.1	30.41±1.8	32.30±2.06	34.67±1.33
WBCs count (×10^{3}cells /µl)	22.43±0.03	22.18±0.19	23.67±0.18	24.0±0.61
Lymphocyte count (×10^{3}cells /µl)	12.19±0.5	13.04±0.23	12.63±0.15	13.45±0.37
Monocyte count (×10^{3}cells /µl)	1.75±0.72	1.4±0.10	1.54±0.26	1.9±0.31
Heterophile count (×10^{3}cells /µl)	6.95±0.12	6.92±0.06	7.87±0.04	7.18±0.11
Eosinophile count (×10^{3}cells /µl)	0.37±0.08	0.41±0.09	0.51±0.14	0.46±0.1
Basophile count (×10^{3}cells /µl)	1.07±0.12	1.12±0.03	1.11±0.08	1.07±0.14

n: number of samples **RBCs**: Red blood cell count **PCV**: Packed cell volume
TLC: Total leukocytic count **MCV:** Mean corpuscular volume.
MCHC: Mean corpuscular hemoglobin concentration **WBCs**: White blood cell count
Significant at P≤0.05

RBCs osmotic fragility results

The RBCs osmotic fragility, at the appearance of symptoms, there was a significant increase in in gps. (3 and 4) represented by initial lysis at (0.59± 0.04%) saline conc. with complete lysis at (0.32 ± 0.02%) saline conc. in group (3) and initial lysis at (0.64±0.06%) saline conc. with complete lysis at (0.34± 0.02%) saline conc. in gp. (4). Gp. (2) showed nonsignificant changes Fig. (1). One week after treatment the RBCs osmotic fragility showed non significant changes in all gps. Fig. (2).

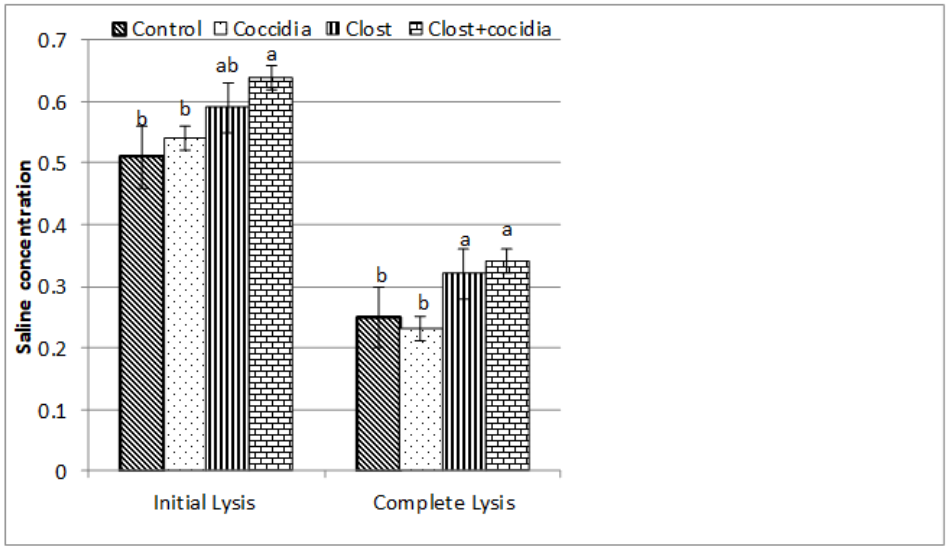

Fig. (1): Osmotic fragility test of the control and tested groups (Mean±SE) (n=5) at the appearance of symptoms.

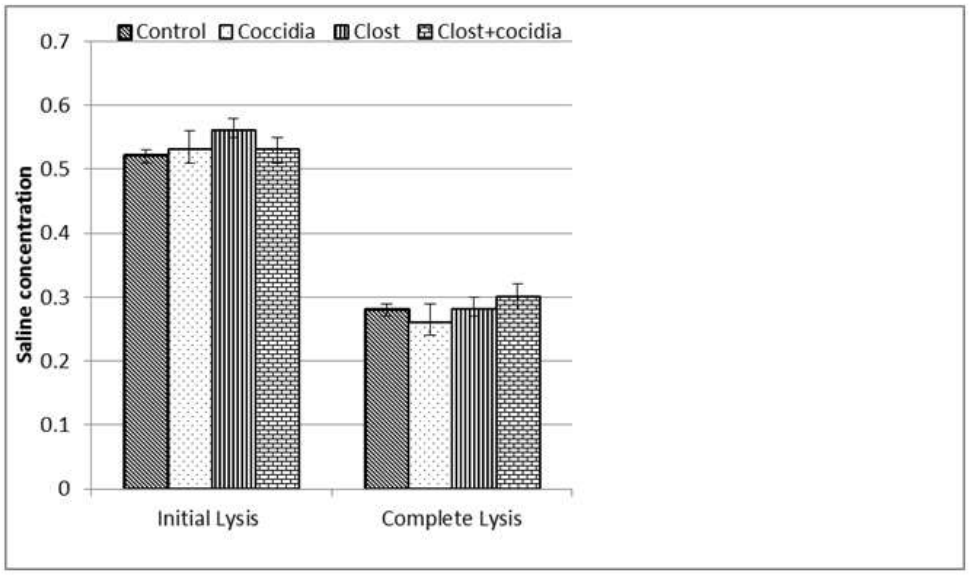

Fig. (2): Osmotic fragility test of the control and tested groups (Mean±SE) (n=5) after treatment.

DISCUSSION

Intestinal coccidiosis and necrotic enteritis are implicated in severe economic losses in poultry industry. So the use of antimicrobials has been recognized as the most important turkey health issue. Prerana *et al.* (2018) found that amoxicillin was most effective against (85.71%) of tested isolates. Other studies performed in the United States, China, and Norway had suggested that amoxicillin is the most effective against *C. perfringens* infection in poultry (Lianco *et al.*, 2012). In addition to that El-Banna *et al.* (2005) and El-Dakhly

et al. (2006) who reported that diclazural in the drinking water was the best choice for treatment of *Eimeria* spp.

Regarding the hematological changes associated with coccidiosis and necrotic interitis, a significant decrease in RBCs count, Hb content, PCV and MCHC with a significant increase in the MCV were seen in gps. (2 and 4) at the appearance of symptoms indicate macrocytic hypochromic anemia and the nonsignificant change in MCHC in gp. (3) indicate macrocytic normochromic anemia, which may be hemorrhagic or hemolytic anemia, but the increase in platelets count gps. 2 and 4 experimentally infected with Coccidia and Coccidia and *C. perfringens* respectively may indicate hemorrhagic anemia due to coccidial infestation to the intestinal mucosa. On the other hand the increase in the RBCs osmotic fragility in gps. 3 and 4 represented by the increase in the saline concentration of the initial lysis and complete lysis may indicate hemolytic anemia.

This agrees with Soad Belih *et al.* (2015), Melkamu *et al.* (2018). In addition to Mohammed (2012) who mention that the decline in the blood components in coccidiosis may be due to the severe bleeding and tissue damage in the mucosa of duodenum originated from invasion of *Eimeria tenella* (Mohammed 2012). Moreover, Melkamu *et al.* (2018) reported that, this due to the severe bleeding and tissue damage in the mucosal surface of intestine occurred at acute stage of infection from the invasion of different *Eimeria* spp.

The hemolytic anemia in gps. 3 and 4 infected with *C. perfringens* and *Eimeria spp.* and *C. perfringens* respectively may be due to liberation of large quantity of histamine as a result of the tissues injury which caused increased permeability of capillaries and venules allowing exudation of large quantities of fluid. The macrocytic normochromic anemia in gp. 3 infected with *C. perfringens* that agree with Soad Belih *et al.* (2015). This may be also due to Alpha toxins of the *C. perfringens* type A which are considered to be the major toxins involved in the disease pathogenesis. The activity of alpha toxin is lethal, necrotizing and hemolytic (Quinn *et al.,* 2004; Fatmawati *et al*., 2013). The hemolytic activity may be due to binding of alpha toxin on the red cells receptor, which activates the signaling pathway in the cell, resulted in the hemolysis. Our finding is supported by Ombe *et al.* (2006).

Regarding the leukogram, the heterophilic leukocytosis in birds of gps. 3 and 4 may be due to bacterial infection but the eosinophilia in gp. 2 and 4 may be due to the parasitic investigation by the *Eimeria spp.* as mentioned by Melkamu *et al.* (2018). This was previously reported by Feldman *et al.* (2000) who mentioned that heterophilia is frequently observed with tissue damage induced by inflammation or bacterial infection, but eosinophilia in birds is commonly observed with parasitism. Tully *et al*. (2000) reported that leukocytosis and hetrophilia often indicate presence of infection or cellular damage and the same obesrved by Coles (1997), who also mentioned that an increase in the eosinophil count in avian blood samples indicate parasitic infection. The heterophils infiltration increase immediately after any infection as a first line of defence followed by increase in eosinophil concentration as a response to parasitic infection (Wakenell, 2010). Basopenia in gp. (4) may be of limited clinical importance in avian *spp.* as in other *spp.* as reported by Feldman *et al.* (2000). The Nonsignificant changes lymphocytic and monocytic counts in this study disagree with Melkamu *et al.* (2018) who observed increased numbers

of lymphocytes on broilers infected by *C. perfringens and E. tenella*, in both infections.

One week after treatment with diclazuril, amoxicillin and their combination in gps 2, 3 and 4 respectively showed improvement of the blood picture, whrer hemogram, RBCs osmotic fragility in addition to leukogram as total and differential leukocytic counts were significantly unnchanged, these result agree with Sanaa *et al.* (2020) who treated coccidiosis and NE in turkey with diclazuril and amoxicillin, El-Banna *et al.* (2005) and El-Dakhly *et al.* (2006) who reported that diclazural in the drinking water was the best choice for use in treatment of *Eimeria spp*., Aboubakr and Elbadawy (2017) who used Amoxicillin in Controlling Clostridium perfringens infection in Broiler Chickens and Palmer (1976) who reported that Amoxicillin is a one of most effective β lactam antibiotic.

CONCLUSION

The obtained data from this study differentiated between the type of anemia in cases of coccidiosis and Necrotic enteritis in turkey. It declared that coccidiosis produced hemorrhagic anemia, but *C. perfringens* infection caused hemolytic anemia and necrotic enteritis caused mixed type of hemorrhagic and hemolytic anemia. It also studied the changes in the leukogram associated with it. In addition to that, amoxicillin and diclazuril in therapiotic doses ameliorated the changes associated with coccidiosis and necrotic enteritis in turkey. We recommended that, the use of diclazuril and amoxicillin and good management are important for control and prevention of coccidiosis and necrotic enteritis in turkey.

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التغيرات الهيماتولوجيه المصاحبه للعدوى المشتركه بالكوكسيديا والالتهاب المعوى النخرى فى الرومى

دعاء ابراهيم احمد مصطفى ، سناء محمد سالم
E-mail: doaaibrahim706@gmail.com Assiut University web-site: www.aun.edu.eg

يعتبرالتهاب الامعاء النخرى من اهم المشاكل فى صناعه الدواجن عالميا ومحليا حيث تتسبب اصابة كتاكيت الرومى بالكوكسيديا والكلوستريديم فى خسائر فادحه فى مزارع تسمين الرومى. ونظرا لوجود القليل من المعلومات عن تطورات المرض وتأثيراته على قطعان الرومى خاصا فى مصر، لذلك تهدف هذه الدراسه لعمل نموذج تجريبى للاصابه بالكوكسيديا والتهاب الامعاء النخرى فى الرومى وذلك لتحديد التغيرات الهيماتولوجيه المصاحبه لهم ومحاولة معرفة كفاءة الاموكسى سيللين والديكلازوريل فى التغلب على هذه التغيرات.

ولاجراء هذه الدراسه استخدمنا ٤٠ كتكوت رومى، تم تقسيمهم عشوائيا الى 4 مجموعات متساويه. المجموعه الاولى تركت كمجموعه ضابطه سلبيه، الثانيه تم اصابتها تجريبيا بالكوكسيديا، الثالثه تم اصابتها تجربيا بالكلوستردیا و الرابعه تم عمل عدوى مختلطه بالكوسيديا والكلوستردیا وقد تم علاج المجموعات الثانيه والثالثه والرابعه بالديكلازوريا والاموكسيسيلين والاثنين معا على التوالى بعد ظهور الاعراض.

بالدراسه التجريبيه وجد ان العدوى بهذان المرضان ادى إلى حدوث انيميا المجموعات الثانيه والثالثه والرابعه مع زياده فى عدد الصفائح الدمويه فى المجموعتين الثانيه والرابعه وزياده فى هشاشة كرات الدم الحمراء فى المجموعتين الثالثه والرابعه بالاضافه الى زياده فى عدد كرات الدم البيضاء الكلى والنوعى فى كل المجموعات المصابه. هذا وقد وجد ان الاموكسيسيلين والديكلوسول لهما تاثير كبير وفعال فى التغلب على هذه التغيرات.