Molecular identification of Eimeria tenella in broiler chickens in Kalyoubia governorate and evaluation of different strategies for control cecal coccidiosis.

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A B S T R A C T

Ceacal coccidiosis one of Nine *Eimeria species* affecting chickens and results in severe economic losses. Sixty-seven GIT sample were collected from broiler flocks showing bloody diarrhea in Kalyoubia governorate. 88% of collected samples were found to be positive for cecal coccidiosis after using microscopical and molecular identification. Different control strategies as diclazuril, vaccination (coccivac B® and isolated strain as field strain vaccine) and the aqueous extract of neem plant (*Azadirachta indica*) were used to control cecal coccidiosis in experimentally infected broiler chicks. The results showed that the diclazuril was the best protection method as there was a significant improvement in performance parameters, significant drop in oocyst shedding, dropping scoring, lesion scoring and cecal mucosal scraping scoring. Also, there was minimal histopathological alteration in the cecum of infected broilers in comparison with other treatments. The neem extract treated group also recorded an improvement in the aforementioned parameters in comparison with the vaccinated infected birds. The vaccination of birds using isolated strain achieved better protection against coccidia infection than the imported strain.

Key words/ E. tenella, PCR, neem (*Azadirachta indica*), coccivac B®, diclazuril

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1. INTRODUCTION

Poultry coccidiosis is a parasitic disease cause severe losses in poultry production. It multiplies in the intestine causing severe tissue damage that lead to lower feed intake, poor absorption of nutrients, dehydration, and blood loss in addition to secondary bacterial infections. In spite of the use of medication, coccidiosis generally remains a subclinical disease that affect in performance (Fanatico 2006). Ceacal coccidiosis one of Nine Eimeria species affecting chickens and results in severe lesion of caeca, body weight loss, hemorrhagic diarrhea and death (Witcombe, Smith et al. 2014) E. necatrix makes its schizogony in the middle of the intestine but gametogony occurs in the caecum so that the caecum is the predilection site for isolation oocysts E. tenella and E. necatrix (ZHAO, LI et al. 2010). Identification of Eimeria species based on morphological features of the sporulated oocyst, sporulation time and location require specialist expertise and have serious limitations (Long and Joyner 1984). This led to development of polymerase chain reaction (PCR) assays which is considered as the most accurate molecular diagnostic tool for Eimeria infection (Blake, Hesketh et al. 2006) moreover, the multiplex PCR can make simultaneous amplification of more than one locus in one reaction by using more than one pair of primers (Fernandez, Pagotto et al. 2003). Poultry coccidiosis mainly controlled by the hygienic measures, anticoccidial drugs and vaccines (Soulsby 2015). Wherever poultry flocks are raised and drugs are used to combat Eimeria infections, drug resistance problem may occur (Chapman and Jeffers 2014). Recently, resistance of coccidia against all anticoccidial drugs has been developed also consumers now request poultry products that are free from drug residues (Abbas, Iqbal et al. 2010). Vaccine is promising tool for control coccidiosis without leaving any residues. On the other hand, vaccination are unstable, high economic cost and inefficient in controlling a large number of coccidian strains which prevalent in different geographical areas due to antigenic diversity (Shah, Umar et al. 2014). Azadirachta indica (neem plant) is old medicinal plants used as anti-helmentic as well as antimicrobial. Moreover, Addition of 0.3% ground neem fruit or aqueous extract of leaves in broiler feed or water had

showed significant reduction in coccidial oocysts output (Nidaullah 2010).

The aim of this study was to make accurate diagnosis of Eimeria species and evaluate the different control strategies to overcome this infection.

2. MATERIAL AND METHODS

2.1. Survey study on Eimeria species affecting broiler flocks

samples collection from 67 broiler showing bloody diarrhea with different age in Kalyoubia governorates during 2014 from different location according to method described by (Duszynski and Wilber 1997). Samples were microscopically examined for presence of coccidial oocyst. Then oocyst Purification and sporulation were carried out according to(Jackson 1964). Eimeria species were identified using multiplex conventional PCR. Coccidial oocyst were destructed by glass beads grinding method described by(Zhao, Duszynski et al. 2001). DNA extracted using Gene jet viral DNA and RNA purification kit (thermo scientific) according to kit protocol. PCR performed using Primers sequence for E.tenella and E.necatrix designed by (Fernandez, Pagotto et al. 2003) at cycling condition according to (Ogedengbe, Barta et al. 2009).To verify the results, 10 µl of each PCR product was electrophoresed in 1% agarose gel, stained with ethidium bromide, and photographed by gel documentation system. The size of PCR products were identified by size using a 100 base pair ladder.

2.2. Experiment.

One hundred and eighty commercial coccidian free unvaccinated one day old broiler chicks (Ross) were housed in a clean well ventilated room previously cleaned and disinfected. Birds were reared in deep litter and kept in a strict isolated mosquito proof room. Anticoccidial free starter, grower and finisher ration were used.

2.3. Experimental design

Birds were divided in to 6 groups each group consisted of 3 replicates 10 bird on each the groups were arranged. Group 1 vaccinated by E. tenella Field strain. Group 2 vaccinated by coccivac B®. Group 3 treated by aqueous extract of neem (Azadirachta indica) leaves. Group 4 treated by diclazuril. Group 5 control positive. Group 6 control negative. At day old Group (1) vaccinated with E.tenella Field strain while Group (2) vaccinated with coccivac B®. Before infection, fecal samples from non-vaccinated groups were examined microscopically to confirm that an absence of coccidial infection. At 21 days of age, all challenged groups received challenge 3×10^3 sporulated oocyst of E. tenella directly to the crop using syring and canula according to (Jatau, ODIKA et al. 2014). Aqueous extract of neem leaves which extracted according to (Nidaullah 2010) and taken by group (3) one day before infection. Group (4) treated with diclzuril four day after infection according to (Assis, Cury et al. 2012). The experiment was terminated after 2 weeks post challenge (35 days of age).

3.1. Incidence of Eimeria species in affected broiler chickens.

2.4. 2.2.2. Evaluation parameters.

Deaths due to coccidiosis were recorded. Mean weight of birds in each replicate were weekly calculated along the duration of experiment. Feed consumption and the feed conversion ratio (FCR) were calculated weekly. Fecal samples from each replicate were collected, and the oocyst per gram of fecal material (OPG) was counted using the McMaster counting technique as described by (Long, Millard et al. 1976). One bird from each replicate was sacrificed 7 days PC. Ceacal lesions were scored according to (Johnson and Reid 1970). Dropping scores were graded (0–4) according (Morehouse and Baron 1970). The ceacal scraping score was graded (0–4) according method described by(Salisch 1987).

2.5. Histopathology.

Ceacal samples from sacrificed birds 7 days post challenge were fixed in 10% formalin solution, and routinely processed for histopathological evaluation (Prophet 1992). The histopathological lesions of each infection were described.

2.6. Statistical analysis

Statistical analysis was performed using the statistical software package SPSS for Windows (version 20.0; SPSS Inc., Chicago, IL, USA). Statistical significance between mean values was set at ($P< 0.05$).Differences between groups were analyzed by using One-Way ANOVA and Duncan's multiple comparison Post Hoc tests (Duncan 1955).

3. RESULT

By microscopical examination of cecal content and cecal mucosal scraping revealed that 88% of the collected samples were positive for *Eimeria* oocyst. And by Molecular identification using

Multiplex conventional PCR revealed that 100% of collected samples give amplicon size 539bp which is the expected amplicon size to *E. tenella* primers which indicate that 100% of these samples were *E. tenella*.

3.2. Effectiveness of different strategies for control challenge of E. tenella infection in broiler chicken.

The recorded mortality rate in group 2 and group 5 were 3.3% and 6.67% respectively. No Deaths recorded in other group.

3.2.1. Feed conversion ratio (FCR).

As illustrated in table (1), FCR Before challenge there was significant increase in FCR in group (1) and group (2) than other groups. After challenge at fourth week, FCR in all groups were significantly increased than group (6) and significantly decreased than group (5) The conclusion of total FCR indicate that there was no significant increase in FCR in group (3) and group (4) in comparison to group (5) but in group (1) and group (2) respectively which significantly increased than group (5) and exceed the level of FCR in group (6).

3.2.2. Oocyst output.

As illustrated in table (2), the oocyst output in all vaccinated and treated groups was significantly decreased than group (5) (control positive group) but the lowest output was in group (4). The oocyst output in group (1) was significantly decreased than output in group (2) and the oocyst output in group (3) was in between the two-vaccinated group with non-significant difference.

3.2.3. Dropping score, ceacal lesion score and Ceacal mucosal scraping score.

As illustrated in table (3), Ceacal lesion score was significantly decreased in all group than group (6) but the lowest score was in group (4) which not significantly different from group (6). Ceacal lesion score was significantly decreased in group (1) but decreased the score in group (2) and group (3) group was not significant statically. Dropping scores in all groups are decreased than group (6) but this decrease was not significant in group (1), group (2) in compare with group (5). Ceacal mucosal scraping score was significantly decreased in all groups than group (6). The highest score was in group (5) followed by group (2) with little static difference.

3.2.4. Histopathological examination

Group (6) No histopathological changes were detected in the examined organs of these chicks. The enterocytes and mucosal crypt epithelium appeared normal and free from various developmental stages and oocysts of Eimeria tenella (figure 3). Group (5) Diffusely, the mucosa was thickened and large numbers of enterocytes (approximately 90%) were swollen and contain intra and extracellular various developmental stages or oocysts of Eimeria tenella (figure4).

Group (1): The histopathological examination of chicks revealed marked lesions nearly similar to lesions of Group (5). Diffusely, the mucosa was thickened and approximately 80% of enterocytes contained developmental stages or oocysts of Eimeria tenella (Figure5). Group (2) The intestinal mucosa was thickened with interstitial leukocytic cellular infiltration particularly lymphocytes and heterophils.

Table (1) Effect of challenge with field strains of E. tenella on feed conversion ratio (FCR) in broiler chickens with different control method.

	Feed conversion ratio (FCR).					
	Before challenge			After challenge		
	1st week	2ndweek	3rd week	4th week	5th week	Total FCR
Group (1)	1.60a±0.01	1.63b±0.06	1.74a±0.05	2.29ab±0.22	1.90a±0.06	1.83a±0.05
Group (2)	1.54ab±0.04	1.93a±0.03	1.78a±0.01	2.17bc±0.24	2.01a±0.16	1.89a±0.06
Group (3)	1.43c±0.03	1.46c±0.02	1.44b±0.00	1.61cd±0.06	1.87a±0.02	1.56b±0.01
Group (4)	1.54ab±0.03	1.44c±0.03	1.44b±0.00	1.75bcd±0.17	1.35b±0.01	1.50b±0.03
Group (5)	1.49bc±0.04	1.48c±0.01	1.44b±0.00	2.77a±0.22	1.93a±0.19	1.82a±0.05
Group (6)	1.51abc±0.02	1.42c±0.01	1.46b±0.01	1.44d±0.08	1.38b±0.06	1.44b±0.01

Each value represents the mean ± standard error. Values with different letters within the same column are significantly different ($p<0.05$).

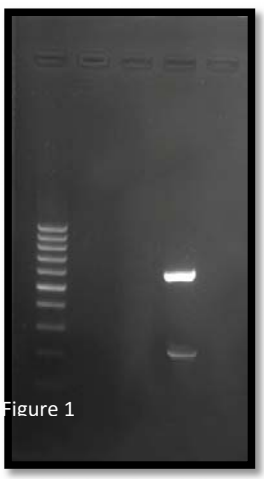
Table (2) Oocyst output in broiler chickens with different control method and challenged with field strains of E. tenella.

	Oocyst output (x103/gram of dropping)							
Group	7th day	8th day	9th day	10thday	11thday	12thday	13thday	Total
Group (1)	$5.60^{b}\pm0.40$	$7.73^{c}\pm0.27$	$8.33^{c}\pm0.28$	$8.20^{c}\pm0.17$	$2.60^{b}\pm0.23$	$1.67^{b}\pm0.34$	$1.07^{a}\pm0.35$	$35.20^{c}\pm1.37$
Group (2)	$6.60^{b}\pm0.67$	$8.37^{bc}\pm0.43$	$10.47^{b}\pm0.41$	$9.70^{b}\pm0.64$	$2.60^{b}\pm0.71$	$1.77^{b}\pm0.24$	$0.87^{a}\pm0.20$	$40.37^{b}\pm1.69$
Group (3)	$7.63^{b}\pm0.54$	$9.03^{b}\pm0.46$	$10.60^{b}\pm0.31$	$7.70^{c}\pm0.26$	$3.17^{b}\pm0.19$	$1.03^{bc}\pm0.09$	$0.10^{b}\pm0.06$	$39.27^{bc}\pm0.59$
Group (4)	$1.17^{c}\pm0.15$	$2.37^{d}\pm0.26$	$3.17^{e}\pm0.22$	$2.80^{d}\pm0.35$	$1.23^{c}\pm0.09$	$0.33^{cd}\pm0.09$	$0.03^{b}\pm0.03$	$11.07^{d}\pm0.41$
Group (5)	$12.40^{a}\pm1.27$	$14.83^{a}\pm0.41$	$19.20^{a}\pm0.61$	$17.07^{a}\pm0.75$	$8.43^{a}\pm0.49$	$2.57^{a}\pm0.38$	$1.20^{a}\pm0.17$	$75.70^{a}\pm2.81$
Group (6)	$0.00^{c}\pm0.00$	$0.00^{e}\pm0.00$	$0.00^{e}\pm0.00$	$0.00^{e}\pm0.00$	$0.00^{d}\pm0.00$	$0.00^{d}\pm0.00$	$0.00^{b}\pm0.00$	$0.00^{e}\pm0.00$

Each value represents the mean ± standard error. Values with different letters within the same column are significantly different ($p<0.05$).

Table (3) Effect of challenge with field strains of *E. tenella* on Ceacal lesion score, dropping score and ceacal mucosal scraping in broiler chickens with different control method.

Group	Ceacal lesion score	dropping score	Ceacal mucosal scraping score
Group (1)	2.33b± 0.33	3.00ab± 0.58	2.67b± 0.33
Group (2)	3.00ab±0.58	3.67ab± 0.33	3.00ab± 0.58
Group (3)	3.00ab±0.58	2.67b± 0.33	2.67b± 0.33
Group (4)	0.67c±0.33	0.67c± 0.33	0.33c± 0.33
Group (5)	4.00a±0.00	4.00a± 0.00	4.00a± 0.00
Group (6)	0.00c±0.00	0.00c± 0.00	0.00c± 0.00



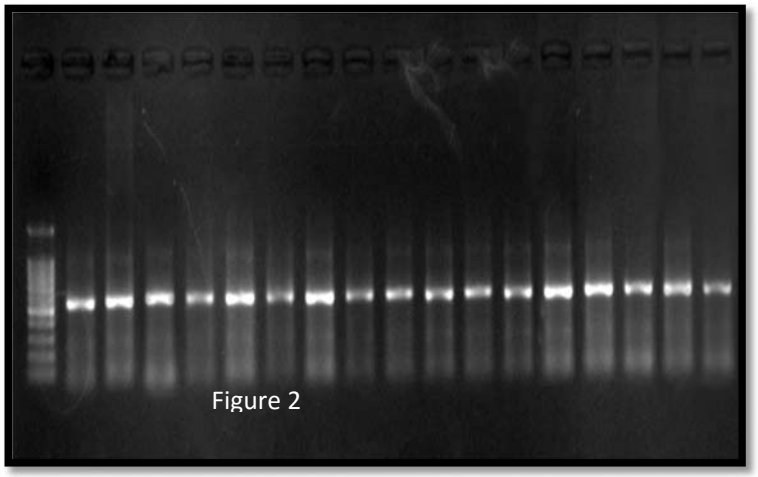


Figure (1): Agarose gel electrophoresis of multiplex PCR products of control positive sample.
Positive reactions: *E. tenella*(539bp) *E. necatrix*(200bp) 1= 100 bp ladder
Figure (2): Agarose gel electrophoresis of *Eimeria* species-specific PCR products.
Positive reactions: *E. tenella*(539bp) 1= 100 bp ladder

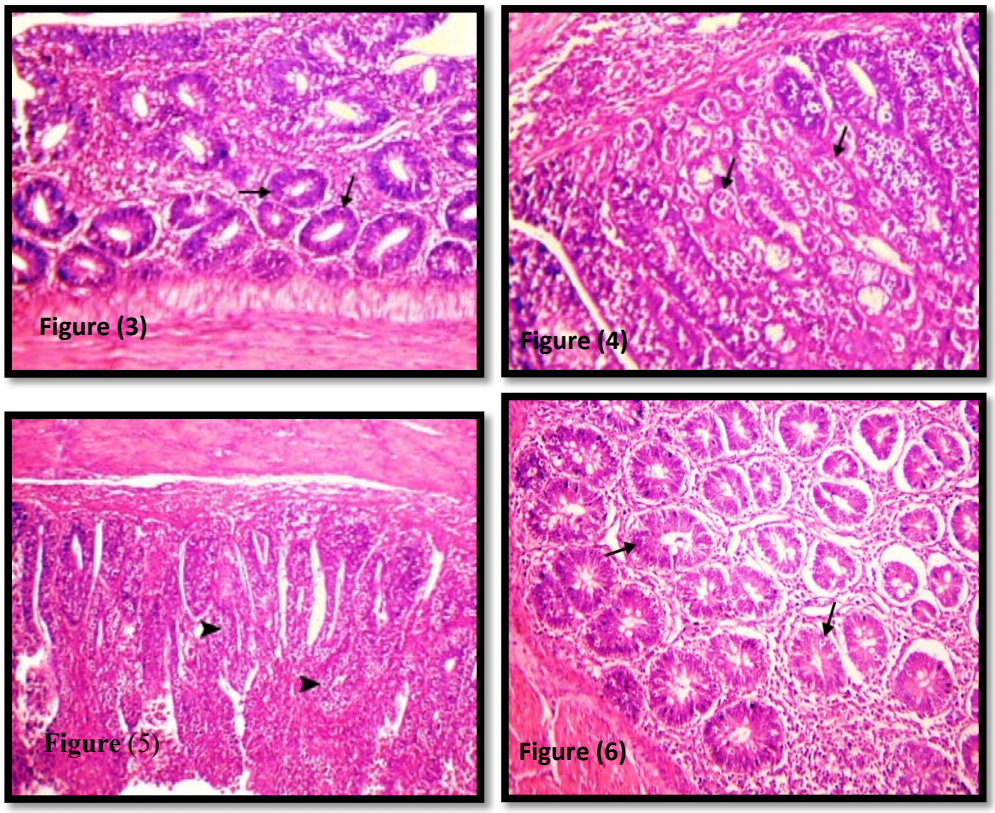


Figure (3): Cecum of control chick showing normal enterocytes and mucosal crypt epithelium (arrow) free from various developmental stages and oocysts of *Eimeriatenella*. H&E stain x 200.
Figure (4): Cecum of chick experimentally infected with acute coccidiosis on 21 days old, showing thickening of the mucosa by large numbers of intra- and extracellular developmental stages (arrow) and oocysts of *Eimeriatenella*. H&E stain x 200.
Figure (5): Cecum of Field-strain vaccinated chick experimentally infected with acute coccidiosis on 21 days old, showing thickened mucosa and marked infection of enterocytes by developmental stages and oocysts of Eimeria tenella(arrow head). H&E stain x 100.
Figure (6): Cecum of coccivac-B vaccinated chick experimentally infected with acute coccidiosis on 21 days old, showing normal histological appearanceof most crypts epithelium (arrow) which were free from developmental stages and oocysts of Eimeriatenella. H&E stain x 200.

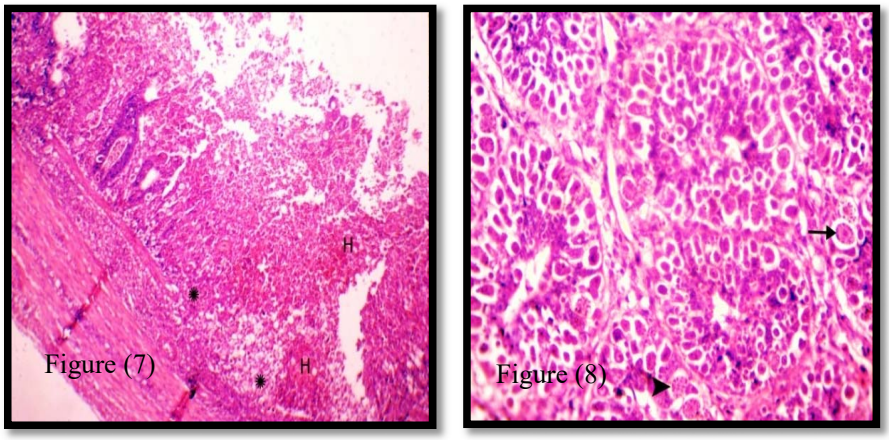


Figure (7): Cecum of chick experimentally infected with acute coccidiosis on 21 days old, and treated by Neem plant showing excessive tissue damage (asterisk), leucocytic cellular infiltration, bleeding (H), with loss of the villi and crypts. H&E stain x 100.
Figure (8): Cecum of chick experimentally infected with acute coccidiosis on 21 days old, and treated by Diclazuril showing macrogamonts(arrow) and microgamonts(arrow head) in the lamina propria and infiltrating the mucosal/crypt epithelium. H&E stain x 400.

Diffusely, large areas of the intestinal villi and mucosal crypt epithelium appeared normal and free from developmental stages and oocysts of Eimeria tenella (Figure 6). Group (3) the caecum of treated chicks showed marked histopathological changes similar to Group (5). Diffusely, enterocytes were swollen and contain various developmental stages or oocysts of Eimeria tenella. These lesions were accompanied by excessive tissue damage, leucocytic cellular infiltration, bleeding, disruption of the cecal glands, or complete loss of the villi and crypts (Figure7). Group (4) the caecum of treated chicks revealed marked lesions in the mucosa and submucosa similar to Group (5). Diffusely, and circumferentially expanding the lamina propria and infiltrating the mucosal/crypt epithelium were numerous intra- and extracellular macrogamonts, microgamonts, and schizonts (Figure8).

4. DISCUSSION

Our results indicate that 88% of collected fecal samples showing cecal coccidiosis. These results were near to those reported by (Mahareek 1992) who recorded that (73.3%) of flocks suspected to be infected with coccidiosis infected with cecal coccidiosis and (Khilfa 1982) recorded the incidence of coccidiosis among chickens in different ages was 84.24% But there is difference between our result and results recorded by (Abd El-Rahman 2003) which show that the incidence of poultry coccidiosis was 68.79%. The development of polymerase chain reaction (PCR) assays provide a necessary tool to accurate diagnosis of Eimeria infection

Our result of the Multiplex conventional PCR indicate that 100% of collected samples give amplicon size 539bp which is the expected amplicon size to E. tenella primers which indicate that 100% of samples is E. tenella. This result was different from those reported by (Shakshouk 1984) who reported that the prevalence of E. tenella were 67.8% and E. necatrix 32.2% and dissimilar to results reported by (Ali 2006) who reported that the prevalence of E. tenella, and E. necatrix in 12 broiler flock were 75%, 17% respectively. Poultry coccidiosis mainly controlled by the hygienic measures and use of anticoccidial drugs also coccidial vaccine. (Soulsby 2015) Other method as addition of aqueous extract of Neem either soley or in herbal plant mixture showed significant reduction in coccidial oocysts (Nidaullah 2010). In the present study, the using of different control methods on birds experimentally challenged with E. tenella resulted in improvement in performance parameters. There is agreement between results recorded from group (2) and result recorded by (Awad, El-Nahas et al. 2013) who recorded that infection of birds in the experimental groups with different strains of E. tenella resulted in a significant reduction in performance parameters (FCR, BW gain, and mortality %) while using of vaccination against coccidiosis improve this parameters.

Drug treated group had lowest oocyst output, highest weight gain, lowest feed conversion rate and best Ceacal lesion score, dropping score and Ceacal mucosal scraping score when compare with vaccinated groups and Neem group so that drug treatment considers the best method for control E. tenella infection. These results agree with the findings of other studies as (Zhou, Wang et al. 2010, Shen, Wang et al. 2012). This result disagreed with the results recorded by (Danforth 1998) who conclude that vaccination with live oocysts elicited significant protection against coccidiosis, similar to that obtained with anticoccidial medication. Also this results disagree with (Suo, Zhang et al. 2006) who conclude that coccidial vaccine Supercox® could control clinical coccidiosis in broilers and achieve production performance better than medicated with Diclazuril. These differences may be attributed to drug and vaccine resistance might result in failure to control clinical diseases. Broiler chicken at group 3 that consume aqueous extract of Neem leaves in drinking water showed improvement in performance parameter when compare with vaccinated groups. These findings suggest that aqueous extract of Neem leaves was better than vaccination for controlling E. tenella infection. The findings observed in this study mirror those of the previous studies of (Nidaullah 2010) that have examined the effect of aqueous extract of Neem leaves on poultry coccidiosis. (Hady and Zaki 2012) compared incorporation dried Neem in broiler diets with salinomycin sodium. The results suggested that 10% Neem incorporation in broiler diet was able to alleviate the adverse effect of E.tenella infection but with lower than drug. These results agree with the findings of our studies and other studies as (Tipu, Akhtar et al. 2006).

Broiler chicken at vaccinated groups showed low improvement in performance parameter when compare with drug treated group and Neem group. These results detected low effectiveness of vaccination in control of coccidiosis. This view was supported by (Zhou, Wang et al. 2010, Shen, Wang et al. 2012) who concluded the effectiveness of drug in control of coccidiosis. Within the

vaccinated groups, there was difference in level of protection as vaccination with E.tenella Field strain led to good protection level than vaccination with coccivac B®.These findings further support the idea of vaccination are inefficiency in control a large number of coccidian strains which are prevalent in different geographical areas (Shah, Umar et al. 2014).

Conclusion: our results concluded that. 88% of collected samples showing cecal coccidiosis which can be controlled by different strategy as drug treatment, vaccination and using of herbal plant. In this study, we observed variable degrees of success of these strategies in protection of birds that were infected with E. tenella strain. Drug treatment had the best protection level followed by using of herbal plant which resolves the problem of drug resistance and drug residue while coccidial vaccine given not acceptable level of protection in broiler chicken when compare with drug treatment and herbal plant Use vaccination strategy using of our native strains give higher protection level than imported strain.

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