ISSN 1682-8356
ansinet.org/ijps

INTERNATIONAL JOURNAL OF

POULTRY SCIENCE

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International Journal of Poultry Science 12 (3): 157-163, 2013
ISSN 1682-8356

Efficacy of *Eimeria tenella* (Oocyst and Sporozoite) Proteins as a Vaccine in Broilers Against Coccidiosis

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Abstract: The protective efficacy against homologous challenge in chickens evaluated by using two proteins of a (Sporozoite) and (Oocyst) from *Eimeria tenella*. Immunization was applied on 3rd and 16th day of age subcutaneously with this two types of protein in separate groups at dose (25µg per chicken). Vaccinated birds were challenged at 30th day of age, demonstrated that sporozoite protein could provide chickens with protection rate around 99.2-99.5%, while oocyst protein gives 67-69% protection, number of oocysts and cecal lesion from chickens in the immunized groups with sporozoite protein decrease significantly and this protein was more effective from another groups. The body weight gain not affected (higher) in sporozoite immunized groups when compared with oocyst protein immunized groups and control positive groups, also we estimated ACI which was demonstrated that sporozoite protein was very effective while the oocyst protein was slightly effective.

Key words: Vaccine, sporozoite, oocyst, *Eimeria tenella*

INTRODUCTION

Eimeria tenella is an apicomplexan parasite which causes coccidiosis in the chickens, represents a severe problem for the poultry industry throughout the world due to the losses from mortality and morbidity (Williams, 1999). *Eimeria* infects the epithelial cells of intestinal lining. Pathological changes may occur by this obligate intracellular pathogen, these change differ from destruction of local mucosal barrier and underling tissues to systematic effects such as blood loss, shock syndrome and ever death (Vermeulen *et al*., 2001). The disease is controlling today by preventive medication using polyether ionophores or chemical agent as anticoccidial drugs, Long usage promotes the development of drug resistance and cause great losses in the poultry industry, it also cause large concerns for public about the chemical residues in food (Vermeuleun, 1998; Allen and Fetterer, 2002; Williams, 2002). Coccidiosis is highly immunogenic. primary infections can stimulate solid immunity to homologous challenges. Therefore, it would seem obvious that vaccines could offer excellent alternatives to drugs (Allen and Fetterer, 2002). So, there are many procedure to control coccidiosis involving immunological, biotechnological and genetically methods of these, immunological approach is considering more important, live vaccines containing virulent or attenuated strains of *Eimeria* are available but their use is limited in poultry industry due to its high cost. Additionally these vaccines consist of several *Eimeria* species, makes them labour as well as intensive cost to produce (Vermeuleun, 1998). Also, these types of vaccine may reverting back to a pathogenic form (Sharman *et al*., 2010). Therefore, our research efforts have been invested in the development of anticoccidial protein vaccines composed of antigens as an alternative to live vaccines. Since the sporozoite was believed to be the target for protective immunity (Brothers *et al*., 1988; Danforth and Andew, 1987). This was taken in our consideration, the present study use the oocyst and sporozoite extract as a vaccine to protect broilers from *Eimeria tenella* parasite.

MATERIALS AND METHODS

Parasite propagation and oocyst, sporozoite protein preparation: Local isolation of *Eimeria tenella* were obtained from (Dr. Katranji M.M., Parasit Lab./Collage of Veterinary medicine/Hama/Syria) and propagated throughout 3 weeks old chickens (Broiler, Ross. 308), Oocysts were collected from the ceca of infected chickens at 7th days post infection. After sporulation with potassium dichromate at 28°C for 6-7 days, oocysts were purified by standard salt flotation techniques and sterilized by sodium hypochlorite treatment as described previously (Schamatz *et al*., 1984). Sporulated oocysts Pic. (1) were stored in phosphate buffer salin (PBS PH = 7.6) at 4°C until further use.

Preparation of sporozoite protein: Sterile sporulated oocysts about 2 ml (4 x 10^7) were used for excystation of sporozoites. Sporocysts were released from their oocysts by vortex with 3.3 gm of glass beads contained in glass vial for about 2-3 min. The released sporocysts

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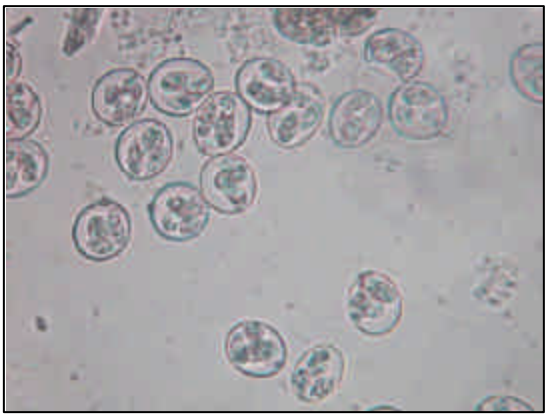
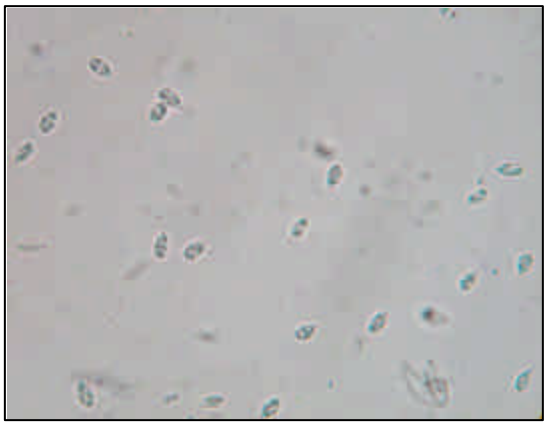
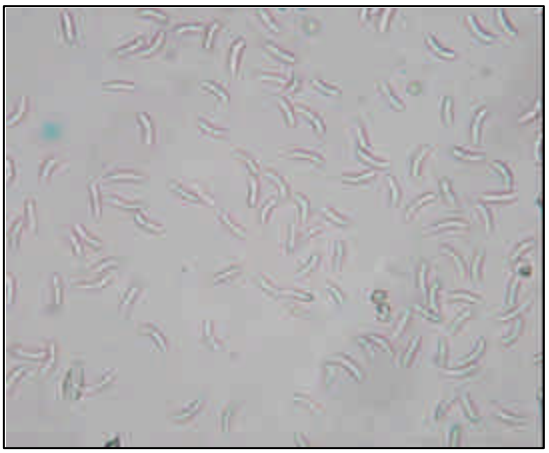


Pic. 1: Sporoulated Oocysts (40x)



Pic. 2: Sporocysts after cracked oocysts(40x)

Pic. (2) were separated from the glass beads by washing with PBS and suspension has been contained sporocysts, oocyst walls and a few intact oocysts. The suspension was centrifuged at 2500 rpm for 3 min. The pelleted sporocysts were suspended in excystation fluid (0.25% trypsin, 5% chicken bile) (v/v) in PBS pH = 7.6 and placed in shaking water bath at 41°C for 3 h. When the majority of the sporozoites had excited, the excystation halted by 3-fold dilution with PBS at 2500 rpm for 3 min. The pellet containing sporozoites, sporocyst walls, unexcysted sporocysts and some intact oocycts was then suspended in PBS. Purification of sporozoites were carried out by use either percoll gradient as described previously by Dulski and Turner (1987) or by use ion exchange chromatography (Schamatz *et al*., 1984; Riggs and Perryman, 1987). Purified sporozoites were counted by use haemocytometer then stored in 1 ml PBS at -75°C until use. Solubilization of purified sporozoites Pic. (3) were carried out by using 100 µl of lysate buffer (0.5% Nonidet P40, Tris-HCL 10Mm, Aprotinen 0.1 U/ml, 1% Triton X -100) which was added to the pelleted of purified sporozoites for 24 h at 4°C with vortex. Then centrifugation at 10000 rpm for 10 min and the supernatant were taken as source of protein (vaccine). Concentration of protein were determined by the method of Bradford assay (Wallach *et al*., 1994).



Pic. 3: Sporozoites after excystation (100x)

Preparation of oocyst protein: About 2 ml (4x 10^7) of purified sterilized oocysts as mention previously were vigorously mixed with glass beads for 10 min. on vortex, then glass beads washed with minimal amount of PBS. The suspension of oocysts, sporocysts, sporozoites and walls was Frozen -196°C in liquid nitrogen and defreeze in water bath at 45°C for 3 times. Lysate buffer were added to the suspension (200 µl/1.5 ml) and incubated for 24 h at 4°C with vortex. Centrifugation was done for the suspension at 2000 rpm for 10 min and the supernatant was taken as a source of protein (vaccine). Concentration of protein were determined by the method of Bradford assay (Wallach *et al*., 1994).

Chickens field experiment : 160 chicks of Broiler (Ross 308) at age of one day-old, coccidiosis free, were obtained from (Hama, Syria) hatcheries. The source of drinking water from main supply and feeding on non medicated broiler diet (according to animal nutritional requirement of local feed tables) (Kussibati *et al*., 2003) as mash *ad libitum*. Throughout the study birds were maintained in 7 separated floor pens, and housed on litter composed of wood shaving of 5 cm depth, temperature in the floor pens was maintained 20-30°C. Extreme care was taken to avoid accidental exposure chicks to coccidia during immunization period and feces were examined periodically by the flotation technique for the absence of coccidial oocysts. The birds were grouped (20-30 chicken per group) at first day of hatch as Table 1.

Table 1: Types of groups used in the experimental design

Groups	Type of groups
G1SA	Vaccinated with Sporozoite protein+Adjuvant, challenged group (20 Birds)
G2S	Vaccinated with Sporozoite protein, challenged group (20 Birds)
G3OA	Vaccinated with Oocyst protein+Adjuvant, challenged group (20 Birds)
G4O	Vaccinated with Oocyst protein, challenged group (20 Birds)
G5	Vaccinated with Adjuvant, challenged group (20 Birds)
G6	Unvaccinated, challenged group (30 Birds)
G7	Unvaccinated, Unchallenged group (30 Birds)

Table 2: The protective efficacy to Some parameters of the used proteins in immunized chickens

	Type of vaccine						
Control	Negative G7	Positive G6	Adjuvant G5	G1SA S-P+adj.	G2S S-P	G3OA O-P+adj	G4O O-P
Oocyst score[a]	0	40	40	5	5	20.0	20
Cecal lesion score[b]	0	3	2.4	0.73	0.64	0.96	1.2
Percentage of protection (%)[c]	100	0	45.5	99.2	99.5	67.6	69.5
ACI index[d]	200	100.2	108.9	183.2	184.3	159.0	154.1

[a]Oocyst score of each group was determined by the calculation and the criteria as described: O.P.G. of the vaccinated group in relation to the control group = (O.P.G. of the vaccinated challenge group) ÷ (O.P.G. of the unvaccinated challenge group) 100: Criteria: 0 to 1%+5: 1.1 to 25%+10: 26 to 50%+20: 51 to 75 %+40: 67 to 100% (Kodama *et al*., 2006).

[b]Cecal lesion scores were determined as average 7, 8, 9 days after challenged with *E. tenella* according Johnson and Reid (1970).

[c]Percentage protection = (the number of oocysts from unvaccinated challenge group - the number of oocysts from vaccinated challenge group) / the number of oocysts from unvaccinated challenge group x 100 (Li *et al*., 2012).

[d]ACI index = (Relative weight gain rate + survival rate) - (Lesion score x 10 + oocyst value). Criteria: 180 or higher: very effective; 160 to 179: considerably effective; 120 to 161 slightly effective; less than 120: not effective (Geriletu *et al*., 2011; Yang *et al*., 2012).

Table 3: Some parameters of the used proteins in immunized chickens which estimated to the period before challenge (28th day of chicken age) to the end of experiment (39th day of chicken age)

	Type of vaccine						
Control	Negative G7	Positive G6	Adjuvant G5	G1SA S-P+adj.	G2S S-P	G3OA O-P+adj	G4O O-P
Average weight gain (g)[A]	839	581	612	802	803	714	723
Relative weight gain (%)[B]	100	70.2	72.9	95.5	95.7	88.6	86.1
Average feed intake (g)[C]	2212	1940.6	2100	2218.7	2235.9	2148.8	2150.4
Percentage feed conversion (%)[D]	37.9	29.9	29.1	36.1	35.9	33.2	33.6
Survival rate (%)	100	100	100	100	100	100	100
Average OPG x (10)[4] 7, 8, 9th day	-	7.0966	3.8675	0.0566	0.03	2.2983	2.16

(A) Average weight gain = Weight of each group at 39 day age of chickens subtracting weight of the same group at 28 day age of chicken.

(B) Relative weight gain (%) = {weight gain of the vaccinated group} ÷ {weight gain of unvaccinated unchallenged group} 100.

(C) Average feed intake (g) = {Amount of feed consumption in each group at period} ÷ {mean of the chickens number in the same group at this period}.

(D) Percentage feed conversion (%) = {Average weight gain of each group at period} ÷ {average feed intake of the same group at this period} 100.

Immunization: A total number of 160 chicks of broiler (Ross, 308) at age one-day old were divided as Table 1 into 7 groups. Groups (G1SA, G2S, G3OA, G4O) were immunized subcutaneously (S/C) on the neck with two doses: first dose at 3rd day of age with 25 µg antigen (sporozoite protein or oocyst protein) emulsified in Freund' s Complete Adjuvant (FCA-Sigma, St Louis USA) (or without Adjuvant) and booster dose was given at 16th day of age with 25 µg antigen (sporozoite protein or oocyst protein) emulsified in Freund' s Incomplete Adjuvant (FICA-Sigma, St Louis USA) (or without Adjuvant). Chicks in group (G5) were inoculated (S/C) with FCA emulsified in PBS as first dose and booster with FICA emulsified in PBS. After two weeks of last immunization an oral inoculation with 10^4 of virulent *Eimeria tenella* sporulated oocysts for all groups except (G7) which kept as unimmunized unchallenged control. Chicks in group (G6) challenged only but didn't immunized.

Evaluation of immune protection: The protective efficacy of the proteins used in immunized groups were measured according Table 2 and 3 to the: Cecal lesion scores which were determined at 7th, 8th, 9th days after chickens being challenged with sporulated oocysts of *E. tenella* and three chicks from each group were chosen randomly then euthanized. The caecum of each bird was examined, the gravity of lesions were scored between 0

and 4 according to the method of Johnson and Reid (1970). Oocysts output, also measured, by taking feces from each group separately between 7 and 9th days post challenge and the numbers of oocysts per gram feces were calculated using Mc Master technique as previously described by (Long *et al*., 1976). Oocyst score of each group was determined by the calculation and the criteria as following: O.P.G. of the vaccinated group in relation to the control group = (O.P.G. of the vaccinated challenge group) ÷ (O.P.G. of the unvaccinated challenge group) 100: Criteria: 0 to 1%, +5: 1.1 to 25%, +10: 26 to 50%, +20: 51 to 75%, +40 : 67 to 100% (Kodama *et al*., 2006). The Body Weight Gain (BWG) of the chickens in each group was determined weekly and the body weight also determined at end of the experiment subtracting the body weight at the time of challenge (Geriletu *et al*., 2011). Percentage of protection also determined as described by (Li *et al*., 2012). Survival rate also determined and the Anti-coccidial Index (ACI) is a Comprehensive indicator of medicine or vaccine as described by Geriletu *et al*. (2011) and Yang *et al*. (2012) were determined. The parameters of: Percentage of relative weight gain, percentage of feed conversion and average feed intake (g) also determined.

Data (Cecal lesion scores, Oocysts output) were analyzed statistically using ANOVA (Analysis Of Variance) test.

RESULTS

The efficacy of *E. tenella* vaccine of oocyst or sporozoite (summarized in Table 2 and 3) were described as

Oocysts output: Significant decrease in oocysts output between immunized groups G1SA = 0.0566 x 10^4, G2S = 0.03 x 10^4, G3OA = 2.2983 x 10^4, G4O = 2.16 x 10^4 per gram feces (OPG) as compared with unimmunized control G6 = 7.0966 x 10^4 (P = 0.01) and G5 = 3.8675 x 10^4 (P = 0.05) (Fig. 1).

Oocyst score: The oocyst score of each group was determined and show decrease in immunized groups G1SA = 5, G2S = 5, G3OA = 20, G4O = 20 but in unimmunized control groups oocyst score were G6 = 40 and G5 = 40.

Lesion score: The immunized groups had mean lesion score G1SA = 0.73, G2S = 0.64, G3OA = 0.96, G4O = 1.4 which different significantly from unimmunized control groups (p = 0.01) G6 = 3 and G5 = 2.4 (Fig. 2).

Percent protection: We could see percent protection for immunized groups of sporozoite vaccine G1SA, G2S, more effective than in oocyst vaccine G3OA, G4O. (99.2, 99.5, 67, 69%), respectively.

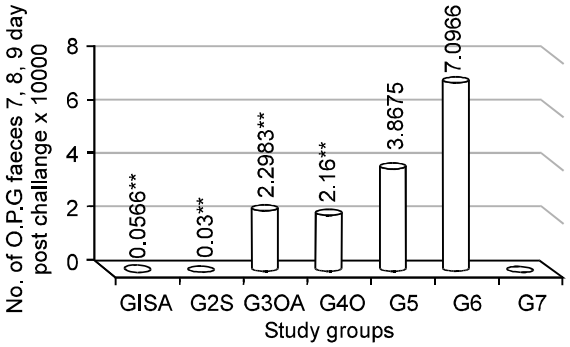


Fig. 1: Oocyst output

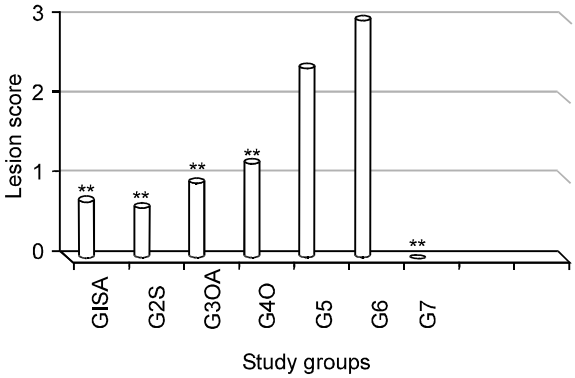


Fig. 2: Average lesion score **p < 0.01, * < 0.05 significantly different from the control groups (G5,G6)

Mortality: There was no mortality in all studied groups.

Anti-coccidial index (ACI): The anti-coccidial index (ACI) for immunized groups of sporozoite vaccine was G1SA = 183.2, G2S = 184.3 very effective but with oocyst vaccine was G3OA = 159, G4O = 154.1 slightly effective, while in control groups G6 = 100.2, G5 = 108.9 not effective (Table 2).

Body weight gain: The body weight gain BWG in each group were determined weekly (5 week) as Fig. 3. There was no differences in BWG after immunization with first and second dose of vaccine in groups at four weeks.

BWG after challenge the birds with *E. tenella*, we saw reduction in BWG in unimmunized control groups G6, G5. As in Fig. 4 which described the body BWG estimated at the period before challenge (28th day age of chicken) and at end of the experimental (39 day age of chicken), we saw reduction in the BWG in unimmunized challenge groups G6 = 581g, G5 = 612g, while unvaccinated unchallenged control G7 = 839g, the BWG of sporozoite vaccine groups well and close the control group BWG which was G1SA = 802g, G2S = 803g. The BWG of oocyst vaccine groups was G3OA = 714g, G4O = 723g less than control group G7.

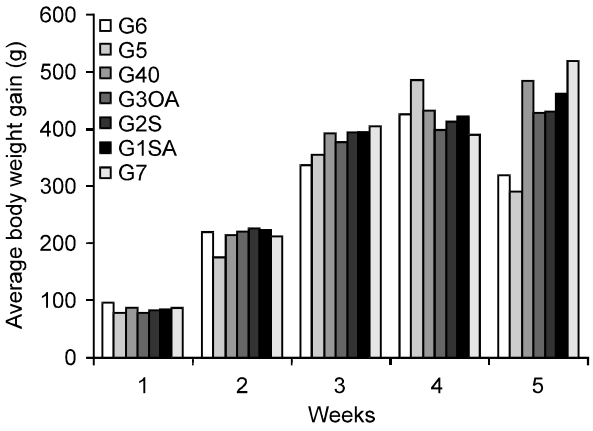


Fig. 3: Average body weight gain (g) weekly

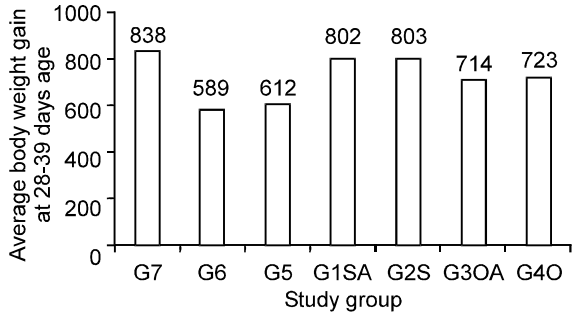


Fig. 4: Average body weight gain (g) for 28-39 day age of chickens

Percentage of relative weight gain: The percentage of relative weight gain in immunized groups was better (G1SA = 95.5, G2S = 95.7, G3OA = 88.9 and G4O = 86.1%), respectively, as compare with unimmunized control groups G6 = 70.2 and G5 = 72.9.

Average feed intake: Average feed intake in grams decrease in unimmunized control groups G6 = 1940.6g, G5 = 2100g as compare with immunized groups were G1SA = 2218.7g, G2S = 2235.9g, G3OA = 2148.8g, G4O = 2150.4g and control groups G7 = 2212.

Percentage feed conversion (%): As compare between control G7 was 37.9 with immunized groups was G1SA = 36.9, G2S = 35.9, G3OA = 33.2, G4O = 33.6 and these except able, while unimmunized control groups less in percentage feed conversion which was G6 = 29.9 and G5 = 29.1.

DISCUSSION

There were very few studies on vaccines prepared against *E. tenella* parasite in Syria and only one study, on live attenuated oocysts vaccine by rays was reported (Al-attar and Al-Qshtiny, 1996). Antigencity of coccidial strains can vary geographically (Allen and Fetterer, 2002) and certain *Eimeria* species may exhibit immunological variation infra specifically (Chapman *et al*., 2005). Live vaccines had many problem related reverting back to a pathogenic form (Sharman *et al*., 2010), high cost preparation and limited period shelf-life. Therefore we use local Syrian isolation to prepare protein vaccines from most important stages in the life cycle of *E. tenella* parasite (sporozoite and oocyst).

No oocysts were detected in the faeces of group G7 chickens throughout this study, demonstrating the success of the procedure adopted to prevent contamination by extraneous coccidia. FCA was found not successful in potentiating immunogenicity of sporozoite and oocyst protein. This result agree with study when use FCA with cell line as vaccine against avian coccidia (Miller *et al*., 1998) but disagree with another study when use FCA with sporozoite protein which was given higher effective (Badawy and Aggour, 2006). The ineffective of FCA in recent study may relative with higher immunogenicity of our used vaccines so there are no differences between groups with adjuvant and without it.

The efficacy of sporozoite vaccine was determined primarily on comparative oocysts output which decrease as compare with control groups or other vaccinated groups with oocyst vaccine ($p = 0.05$). We noticed lower mean of lesion scors for sporozoite vaccine which differs significantly as compared with control groups but not appeared significantly with oocyst vaccine groups. Studies were used sporozoite protein as vaccines reached same results but use different procedure for preparation vaccines (Murray and Galuska, 1986; Karkhanis *et al*., 1991; Badawy and Aggour, 2006). Percent protection for sporozoite vaccines were (99.2, 99.5%) represent substantial degree from control groups and oocyst vaccinated groups of protection. Sporozoite that used as protein vaccine gives 66.7% percent protection (Badawy and Aggour, 2006), while in another studies by Subramanian *et al*. (2008) and Geriletu *et al*. (2011) gives (60, 77.3%), respectively percent protection when use recombinant *E. tenella* sporozoite antigen. In this study the sporozoite vaccine gave more protection and it might be returned to the procedure use (lysis buffer) for preparation and also we use total protein of sporozoite not subunit of sporozoite protein. ACI for sporozoite vaccine had been shown very effective while oocyst vaccine less effective, another study which use sporulated oocysts protein of *E. tenella* gives partially protected chickens (Karkhanis *et al*., 1991) and Geriletu *et al*. (2011) was recorded ACI of DNA vaccine on chickens against *E. tenella* very effective for two types of these vaccine and low lesion scores (1.1, 1.18) but percent protection was (60.4, 65.99%). Average weight gain in vaccinated groups was higher as compared with non vaccinated control groups in the

period of challenge infections and there are no effective in average weight gain among the groups at the immunization period (4 week) when use first and second dose of vaccines. That means our vaccines were improved body weight gain as compare with control positive groups, this is might be due to specific antibody responses as well as cell-mediated responses which were detected in vaccinated groups (Badawy and Aggour, 2006). *Eimeria*, parasite invades the cells of the intestine that producing enteritis and diarrhea, resulting in disability to absorb dietary nutrients through the disruption of the integrity of the intestinal mucosa (Mansori and Modirsanei, 2012) and this lead to loss in weight of infected unvaccinated groups of chickens. Our results agree with another studies were use recombinant vaccine (Geriletu *et al*., 2011; Li *et al*., 2012).

Relative weight gain of immunization groups with sporozoite vaccines were (95.5, 95.7%) higher than oocyst vaccines groups (88.6, 86.1%) and control groups (70.2, 72.9%).

Average feed intake after challenge with *E. tenella* in control groups G5, G6 less than that of control group G7 and vaccinated groups, because of severe infection occur in unvaccinated groups were effected by lesion scores in cecum caused reduction in feed intake. Percentage feed conversion in chickens of sporozoite vaccinated groups was appeared close to the control group G7 but higher to control groups G6, G5 and oocyst vaccinated groups. Our results were showed similarity with study which was use antibody obtained from an egg of immunized chicken with an antigenic outer membrane proteins for sporozoite and merozoit of *E. acervulina*, *E. tenella* and *E. maxima* (Kodama *et al*., 2006). While in another study, there was no correlation between oocyst output, severity of lesions and bird weight gains has been discussed (Williams and Cotchpole, 2000).

Conclusion: Our results demonstrated clearly sufficient protection against coccidia by use the *E. tenella* sporozoite protein as vaccine in broiler after challenge. Further studies are recommended for determination and isolation of immune protective antigens.

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