

EVALUATION OF HUMORAL AND CELLULAR IMMUNE RESPONSES TO *EIMERIA TENELLA* OOCYST PROTEIN AS VACCINE TO BROILER

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ABSTRACT

To determine the type of immune response to oocyst vaccine in broiler against coccidiosis, broilers were vaccinated with two doses of prepared oocyst protein from the local strain of *Eimeria tenella* parasite. The vaccine was applied on 3rd and 16th day of age subcutaneously at a dose (25 µg per chicken), vaccinated birds were challenged at 30 day of age. Blood samples were collected at (7th, 28th and 39th) day of age. The immunogenicity of vaccine was studied by using SDS-PAGE and Western blot. Fourteen polypeptides had been estimated more immunogenic after probing with immunized chicken serum at 39th days of age, their molecular weight are (167.8, 114.5, 83.4, 78.2, 73.5, 53.3, 44.1, 38.8, 36.4, 28.2, 20.5, 18, 14.9 and 13.9) KD. In addition, the levels of γ-IFN and IL-4 were estimated in the serum of immunized chickens by using ELISA kits. The results demonstrated two types of immunity, cellular and humoral responses against *E. tenella* oocyst vaccine.

INTRODUCTION

Coccidiosis in poultry is caused by protozoan parasites of the genus *Eimeria*. Worldwide economic losses due to the parasites have been estimated to exceed 1.2 billion U.S dollars per annum [1]. *Eimeria tenella* is the most virulent species, causing severe hemorrhagic enteritis by infection of the epithelium and the sub mucosa of the ceca and, eventually, death of infected chickens [2].

Many studies have been done to understand the type of immunity to *Eimeria* for controlling this disease. Immune responses to *Eimeria* are complex and involve many facets of

nonspecific and specific immunity, the latter encompassing both cellular and humoral immune mechanisms [3,4]. The lymphocytes, macrophages, and other effector cells act in harmony to secrete cytokines and proinflammatory molecules, mediating the appropriate immune responses to the invading parasite. In contrast to the mammalian cytokines, only a little chicken homologs have been described, the main ones being interferon (IFN)- γ , transforming growth factor (TGF), tumor necrosis factor, interleukin (IL)-1, IL-2, IL-6, IL-8, and IL-15 (LILLEHOJ *et al.*, 2004). Recently, a number of cytokines including IL-17, IL-18, IL-16, IL-12, IL-10, and the Th2 type IL-3, IL-4, IL-13, granulocyte macrophage colony stimulating factor, and IL-5 have been described in chicken [5].

Alterations in lymphocyte subpopulation and cytokine production during *Eimeria* infections in animals have been investigated to clarify the nature of protective immunity [6,7,8].

Various cytokines are produced by macrophages following coccidial infection [9]. T-helper (Th) Th-1 secrete γ -IFN and also tend to secrete IL-2, while Th-2 cells secrete IL-4, and also tend to secrete IL-5, IL-6 and IL-10. The cell type whose secretions dominate may help to determine the outcome of certain parasitic infections [10]. IFN production in chickens has been used as a measure of T-cell responses to coccidial antigens [11,12,13].

Wallach [14] demonstrated the ability of antibodies (raised by live immunization or against purified stage-specific *Eimeria* antigens) to inhibit development of parasite *in vitro* and *in vivo*. There are three principal classes of antibodies known in birds, IgM, IgA and IgY, the presence of other antibody classes such as IgD or IgE in birds has not been documented [15].

The aim of the current study is to investigate the type of immune response when use oocyst of *Eimeria tenella* antigen as vaccine from local strain against coccidiosis, because strain differences in *Eimeria* – induced IFN- γ production were observed [13]. And also to study the type of oocyst antigenicity by western blot technique.

MATERIALS AND METHODS

Parasite propagation:

Local isolate of *Eimeria tenella* was obtained from (Dr. Katranji M.M., Parasit Lab./College 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 the 7th day post infection. After sporulation with potassium dichromate at 28 C° for 6-7 days, oocysts were purified by standard salt flotation technique and sterilized by sodium hypochlorite treatment as described previously [16]. Sporulated oocysts were stored in phosphate buffer saline (PBS PH=7.6) at 4 c° until further use.

-Preparation of oocyst protein (vaccine):

About 2 ml of suspension containing about (4×10^7) of purified sterilized oocysts (Pic. 1). Had previously been 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 at -196 C° in liquid nitrogen and defreeze in water bath at 45 C° for 3 times. Lysate buffer (0.5% Nonidet P40, Tris-HCL 10Mm, Aprotinen 0.1 U/ml, 1% Triton X-100) were added to the suspension (200 µl /1.5 ml) and incubated for 24h. at 4 C° with vortex. Centrifugation was done for the suspension at 2000 rpm for 10 min and the supernatant were taken as a source of protein (vaccine). Concentrations of protein were determined by the method of Bradford assay [17].

- Chickens field experiment:

Eighty chicks of Broiler (Ross 308) at age of one day-old, coccidiosis free, were obtained from (Hama, Syria) hatcheries. The source of drinking water was from the main water supply and the feeding was on non medicated broiler diet (according to animal nutritional requirement of local feed tables) [18] as mash *ad libitum*. Throughout the study, birds were maintained in three separated floor pens and on litter composed of wood shaving to a depth 5 cm. Temperature in the floor pens was maintained at 20-30 C°. Extreme management was taken to avoid accidental exposure of chicks to coccidia during immunization period. In addition 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 hatching as in table (1):

Table (1) Groups of experimental design

Groups	Type of groups
G1	Vaccinated with oocyst protein, challenged group (20 Birds)
G2	Unvaccinated, challenged group (30 Birds)
G3	Unvaccinated, Unchallenged group (30 Birds)

-Immunization:

A total number of 80 broiler chicks (Ross, 308) one-day old were divided into 3 groups (G1, G2 and G3) (Table 1). G1 was immunized subcutaneously (S/C) in the neck with

two doses: first dose at 3rd day of age with 25 µg antigen (oocyst protein), and booster dose was given at 16th day of age with the same dose of protein. After two weeks of last immunization an oral inoculation with 10⁴ of virulent *Eimeria tenella* sporulated oocysts was done for all groups except G3 which was kept as unimmunized unchallenged control. Chicks in group G2 challenged only but were not immunized.

Blood collection:

Blood was collected from the wing vein from all chickens post first dose of 7th days old chicken and after second dose of oocyst protein antigen at 28th days old chicken and also collected at the end of experiment at 39th day of age chicken post challenge. Sera were stored at – 20 °C until use.

Antigens characterization:

E. tenella oocyst antigens were identified by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE).

The extraction of *E. tenella* oocyst protein (50 µg) per lane were lysed by boiling in reducing loading buffer {LB; 25 % glycerol, 5% β-Mercaptoethanol, 10% sodium dodecyl sulfate (SDS), 0.01% Bromophenol blue in Tris /HCl (PH=6.8) 16mM}. Then separation was done by using (SDS-PAGE) on a 5-15 gradient gel. Polypeptide in the gel were transferred electrophoretically to nitrocellulose paper (BA85; 0.4 µM; Schleicher & Schuell, Inc.) in a transblot transfer cell (Bio-Rad Laboratories). Electrophoresis was done with transfer buffer at 4 °C for 1.15 h at constant 250 A. After transferring, the nitrocellulose paper containing the polypeptide was washed two times for 5 min each time, with distilled water. Excess binding sites on the nitrocellulose paper were blocked by washing the paper with Tris buffer saline – tween (TBS-T) PH=7.5 {10mM Tris-HCl, 154mM NaCl and 0.1 % Tween -20} plus to 3% bovine serum albumin, for 24 h at 4 °C.

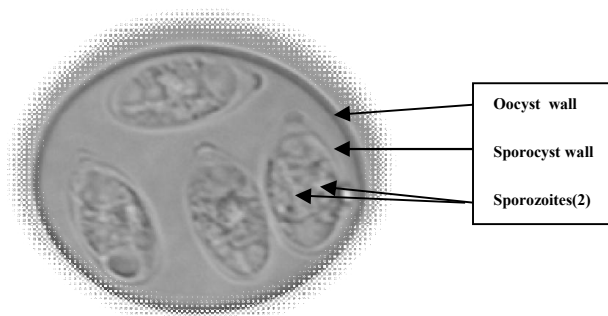
The nitrocellulose paper was further washed 3 times for 5 min. with (TBS-T) buffer on rotator shaker. The membrane was then cut into strips which were separated into individual container and exposed to a 1:200 dilution of the experimental chicken sera in (TBS-T) buffer for 1h. Following washing, the nitrocellulose membrane was incubated for 1h in a 1:1000 dilution of { Rabbit anti – chicken IgG (H&L) conjugated to horseradish peroxidase (Invitrogen Company/California/USA) }, washed (3x5 min) in TBS-T buffer, and finally incubated in OPD (Ortho Phenil diamine) substrate solution for 15-20min with rotated shaker until band had been appeared. The reaction was stopped by washing nitrocellulose stripes for 2 times with D.W

Production of interleukin -4 (IL-4) and interferon -γ (IFN-γ) induced by oocyst protein in chicken:

Serum chicken sample measured at 7th, 28th and 39th days of age with ELISA kits (Life Science Inc. USCNI) for IL-4 and IFN-γ according to the manufacturer's instructions. Optical densities of kit standards and test samples were read at (450 nm) using an ELISA

plate reader (HumaReader HS, Human, Germany). The results were described as pictograms of IL-4 and IFN- γ per 100 μ l of samples.

RESULTS AND DISCUSSIONS



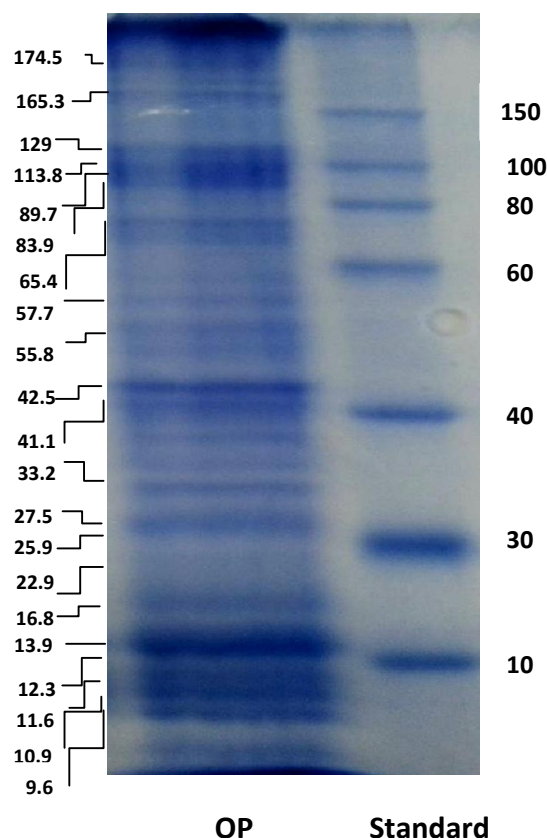
Picture(1): Oocyst of *Eimeria tenella* (100x)

In the current study we prepared oocyst protein from the local strain of *E. tenella* oocysts as a vaccine to immunize broiler. It was generally accepted that asexual stages produce the strongest stimulus for development of immunity [18]. For a better understanding of strain variation is needed for any vaccine to give promising results against local field strains of *Eimeria* [20]. So, the present study has tested the type of immune response against the asexual stage of oocyst protein of local strain of *E. tenella* as a vaccine.

There was no contamination by extraneous coccidian throughout this study, that demonstrated the success of the field experiment.

Antigens analysis

Polypeptides of *E. tenella* were obtained for analysis by the separation of oocyst antigens by SDS-PAGE. There are 21 polypeptides were prominent stained strongly with coomassie blue. Their molecular weight were 174.5, 165.3, 129, 113.8, 89.7, 83.9, 65.4, 57.7, 42.5, 41.1, 33.2, 27.5, 25.9, 22.9, 16.8, 13.9, 12.3, 11.6, 10.9 and 9.6 KD. Pic. (2).



Picture (2): SDS-PAGE(5-15%) stained with Coomassie blue after electrophoretic separation of oocyst proteins(OP).

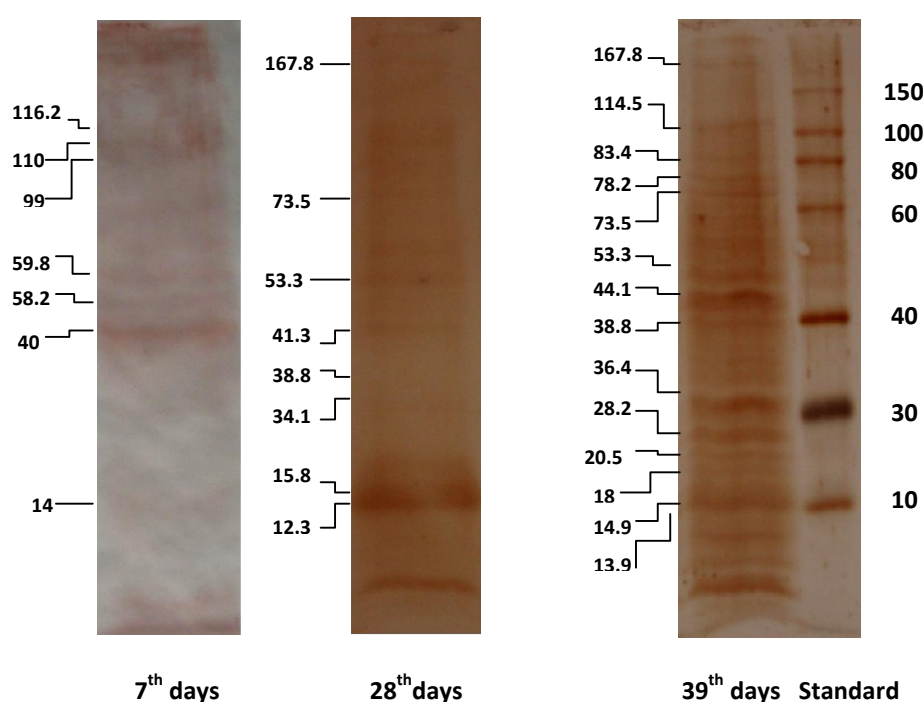
Murray and Glausk [21] detected thirteen polypeptides with MW (235,175, 105, 94, 88, 82, 80, 68, 60, 50, 45, 28 and 26)KD. from oocyst of *E.tenella* by using SDS-PAGA. In comparison. Talebi [22] found eleven polypeptides with molecular weight (116, 84, 66, 58, 48, 45, 36, 24, 22, 20 and 14) KD during his study on *E.tenella* oocyst by use SDS-PAGE.

The apparent differences in molecular size are presumably due to minor technical differences associated with measurement at these sizes with gel [23]. Stotish *et al.* [24] reported that polypeptide was somewhat heterogeneous in molecular size , possibly because of the random cleavage of smaller peptide fragment during solubilization.

In western blot, the reaction of parasite-specific IgG(H&L) antibodies with oocyst antigens is differ when each of 3 immune sera are used as a probe in the immunoblotting procedure . These differences were most obvious between molecular weight 12.3 and 167.8KD Pic. (3). Immunized serum of 39 day age of chicken consistently identified and reacted more intensely with more numbers of antigens in oocyst preparations than immunized serum at 28-7 day of

age of the chicken. These antigens with a molecular weight (167.8, 114.5, 83.4, 78.2, 73.5, 53.3, 44.1, 38.8, 36.4, 28.2, 20.5, 18, 14.9 and 13.9) KD were identified with immune serum post of challenge dose, by *E. tenella* parasite .

The immunized serum at 7 days of age was reacted slightly when used as a probe. Six antigens have been reported with a molecular weight (116.2, 110, 99, 59.8, 58.2, 40 and 14.1) KD .In comparison eight reacted immunized serum were identified at 28 day age. Their molecular weight were (167.8, 73.5, 53.3, 41.3, 38.8, 34.1, 15.8 and 12.3) KD. Immune serum of 28 and 39 days age reacted with five common antigens (167.8, 73.5, 53.3, 38.8 and 15)KD.



Picture(3): Immunoblot of proteins transferred from a 5-15 %SDS-PAGE. Lanes are immunoblots of Oocyst extract preparations probed with immune serum (7th days) :after first dose of vaccine ; (28th days): after second dose of vaccine and (39th days) after challenge with *E.tenella* oocysts.

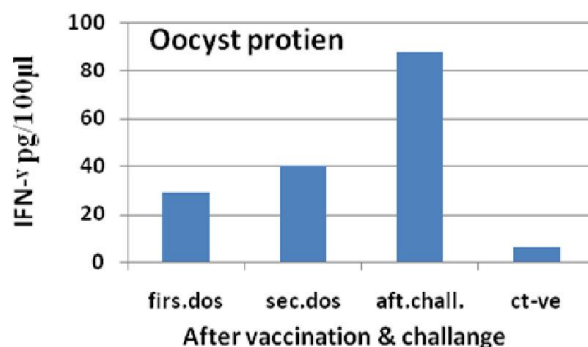
In this study, there were differences in the identification of oocyst antigens and staining intensities among sera that were collected from chickens at (7, 28, 39) days of age. We found that the number of proteins and intensity of reaction was increased after second dose of oocyst antigen and after challenge with *E.tenella* parasite which might be due to the type of using IgG conjugate.

IgM produced in largest amount during primary response, with more IgG being produced during subsequent exposures to the stimulating antigen. Responses are generally stronger and more rapid at second exposure [25]. Tress *et al.* [26] found during his study that IgM is the class of antibody which appears first following *E. tenella* infection before IgA and IgG types of antibody are produced, and serum IgM and IgG were peaking at 13th and 17th days post infections with *E. tenella* parasite. Another study demonstrated that after *E. tenella* infection, significantly higher levels of IgM were produced in the cecum at 7 and 14 days post infection [27]. In contrast Davis [28] reported that the maximal antibody production varies (from 2 to 4 week pi) as humoral antibody responses to avian coccidian which was depended on immunogenicity of the *Eimeria* species, being earlier in infection with *E. maxima* than with *E. tenella*.

Murray and Glausk [21] observed seven polypeptids that reacted strongly with anti sporozoite sera when used oocyst protein as an antigen in western blot analyses, these were 235, 105, 94, 71, 64, 45 and 26)KD molecular weight. This finding indicates that those proteins are strong antigens. Another study found four immunogenic bands (45, 22, 20 and 14)KD in oocyst proteins of *E. tenella* [22]. These proteins were slightly differed from those observed by Hasbullah *et al.* [29] who revealed three common immunogenic bands (23, 21 and 14KD) between oocyst and second generation merozoites of this species. These variations in size of immunogenic protein bands might be due to antigenicity of coccidial strains which vary geographically [30]. In addition, certain strain of *Eimeria* exhibits immunological variation [31] Experiment of Passive immunizations showed a good correlation between the intensity of IgG and IgM antibodies that binding to gametocyte antigens by both western blot and ELISA with the ability of those sera to provide passive protection *in vivo* [17].

Evaluation of IFN- γ and IL-4 production:

After immunization with oocyst protein, IFN- γ and IL-4 levels in the serum samples were examined using an ELISA assay. **The average expression level of IFN- γ** was estimated to be 87.8 pg/100 μ l at day 39, which was more than fifteen fold increase as compared with control negative group (the data shown in fig.1).



Figure(1):Level of IFN- γ in serum of broiler after first vaccine dose(firs.dos),second dose(sec. dos) with oocyst protein and after challenge(aft.chall) with 10^4 oocyst of *Eimeria tenella* .

Li *et al.* [32] found that IFN- γ levels in the serum samples were significantly higher when compared with those of the PBS group using rhomboid- like protein of *E. tenella* as a subunit vaccine in protective immunity against homologous challenge, and also a marked increase in IL-2 level in the serum samples as compared with the control group. Laurent *et al.* [33] showed that IFN- γ expression in the cecum and jejunum of White Leghorn chickens increased more than 200-fold at 7 days post primary infection with *E. tenella* and *E. maxima*. Hong *et al.* [34] supported these observations by finding a significant up regulation of IFN- γ expression in the duodenum of *E. acervulina* infected chickens and ceca of *E. tenella* infected chickens, probably due to the recruitment and stimulation of TCR2⁺ and CD4⁺ IELs (intraepithelial lymphocytes), respectively. Lillehoj *et al.* [15] found during their study on cytokine, it does inhibit *E. tenella* development *in vitro*. In the peripheral blood, a transient but sharp increase in the proportion of CD8-expressing T cell was found in White Leghorn chicken at 8 days after a primary infection with *E. tenella* [35]. This increase was found to be concurrent with a marked increase in interferon IFN- γ and in nitric oxide (NO) production upon *in vitro* stimulation of PBL of T-cell mitogens and *E. tenella* sporozoite antigen [35].

The effect of IFN- γ against *E. tenella* by using immunohistochemistry showed that macrophages were surrounding *E. tenella* sporozoites within 48h after intra cecal inoculation [36]. There is experimental evidence from *in vitro* data that IFN- γ may stimulate macrophage and neutrophils to produce reactive oxygen intermediated which kill *Eimeria* sporozoites [37].

Production of NO₂⁻ + NO₃⁻ and IFN- γ in serum increased during host response to infection of *Eimeria* [38,37].

In this study we, observed high level of IFN- γ production after challenge as compared with control negative group, which increase after first and second dose of vaccine from control negative group. Because host response to the infection after the challenge and the intestinal lesions in coccidiosis are caused, in part. During infection, cytokines such as IFN- γ can stimulate inflammatory cells like macrophage to synthesize highly reactive free radical, NO. These NO are not only toxic the invading parasite but also can damage the host tissue [39]. Therefore, IFN- γ is highly post challenge.

The average expression level of IL-4 was estimated to be 158 pg/100 μ l at day 39, which appeared higher than control negative group which was 41.7 pg/100 μ l (the data shown in fig.2).

The results showed that there were an increase level of IFN- γ and decrease in the level of IL-4 in serum samples after second dose of vaccination.

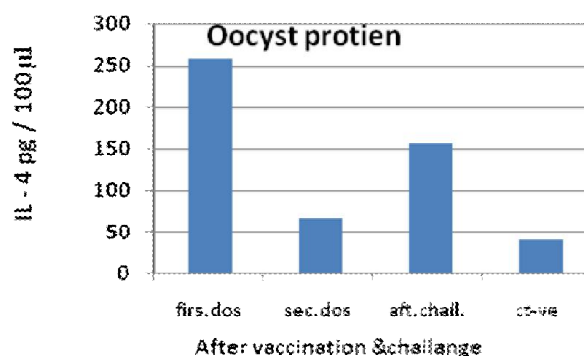


Figure (2): Level of IL-4 in serum of broiler after first vaccine dose(firs.dos), second dose(sec.dos) with oocyst protein and after challenge(aft.chall) with 10^4 oocyst of *Eimeria tenella*.

Chicken Th2 cells are necessary for inducing the humoral response to combat parasite invasion [40,41]. In this study the level of IL-4 in serum of immunized chicken was higher after the first vaccine dose and then drastically decreased after the second dose of vaccination, and return to high level after challenge. Hong *et al.* [34] found during their study on analysis of chicken cytokine and chemokine gene expression following *E.acervulina* and *E. tenella* infections, IL-4 and IL-13 mRNAs were decreased 25 to 2×10^5 - fold after primary and secondary infection.

The differences between IL-4 and IFN- γ might be due to the mutual antagonism of them action, IFN- γ acts on B cells, T cell, NK cells and macrophage. In addition, IFN- γ stimulates B cell to produce of IgG2a and lowers production of IgG3, IgG1b in mice. It enhances T cell production of MHC class I molecules but not the production of MHC class II molecules. It induces Th1 cells to produce both IL-2 and IL-2R. It acts on Th2 cells to inhibit the production of IL-4 and as a result blocks IgE production *in vitro*. IFN- γ enhances the functions of NK cells and activates macrophages and greatly increases their ability destroys ingested microorganisms. It promotes antibody –mediated phagocytosis as well antibody –dependent cell- mediated cytotoxicity (ADCC) reactions. IFN- γ increases MHC class I expression on tumor cell lines and induces the appearance of MHC class II molecules on endothelial cells, and fibroblasts, as well as on macrophages [42].

A previous study on this oocyst vaccine has reported (67-69%) protection, number of oocyst per gram of feces and cecal lesion score from chickens in the immunized groups with oocyst protein decreased significantly from control groups . The body weight gain was not affected, also when estimated anti-coccidia index (ACI) which has been revealed the oocyst protein slightly effective [43].

The immune response to our experimental vaccine demonstrated humoral and cellular protection. Li *et al.* [32] suggested during their studied, specific IgG antibody responses against *E. tenella* was generated in the chickens immunized with recombinant rhomboid like protein expressed in *E.coli* and this protein is capable of eliciting humoral response and activating cell- mediated immunity in birds. Akhtar *et al.* [44] showed the humoral and challenge responses when use supernatant from sonicated sporulated oocyst that induced a strong protection as immune chicks contain high level of antibodies to resist heavy dose of challenge .Similar finding was also observed by [9,14]. .In contrast, another study found the antibody responses have a minor role in protective immunity to *Eimeria spp.* because agammaglobulinemic chickens produced by hormonal and chemical bursectomy are though resistant to reinfection with *Eimeria spp.* [45]. Therefore, Th1 responses seem to be dominant during coccidiosis, as best manifested by proving the involvement of IFN- γ [5]. Subramanian *et al.* [39] found during his study on the (EtMIC1) a recombinant *E. tenella* sporozoite antigen in birds could induce high antibody and strong cell mediated immunity responses. Previous studies agree with this observation when used different antigens [46,47] . In contrast, Wakelin

and Rose [48] found both humoral and cell-mediated mechanisms are involved in anti-coccidial immunity but it has been suggested that the latter plays a major role in protection.

Therefore, different antigens preferentially stimulate different immune responses [49]. Finally it was found that in order to a chief protective immunity by using parasite extracts, it requires the inclusion of the correct antigens and exclusion of the irrelevant ones [17]. This indicates while some parasite-specific antigens induce protective immunity, others actually induce an exacerbation of the infection. Therefore, in the design of any parasitic vaccine, it is crucial that the combination of various antigens maximizes their inhibitory effect of the parasite growth and development [50].

CONCLUSION

oocyst protein vaccine has many immunogenic antigens which reacted more intensely from other protein. We recommend to use these antigens as a vaccine of single band to determine which band has a most immunogenic pattern. In addition during this study, we determined two types of immune responses to our use oocyst protein vaccine to locale Syria isolate of *E.tenella* parasite which was humoral and cellular immune responses.

تقييم الاستجابة المناعية الخلوية واللقاح البروتيني للكيسات البيضية لطفيلي الايميرية في تنلا *Eimeria tenella* دجاج اللحم

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الخلاصة

ترمي هذه الدراسة تحديد نوع الاستجابة المناعية للقاح الكيسات في دجاج اللحم ضد طفيلي الايميرية تنلا. إذ تم تلقيح دجاج اللحم بجريعتين من اللقاح المحضر من السلالة المحلية للايميرية تنلا لطور الكيسات البيضية كبروتين. تم اعطاءه بواقع جرعتين (25 ميكروغرام لكل دجاجة)، في اليوم الثالث والسادس عشر من عمر الدجاج تحت الجلد، واعطيت جرعة التحدي بعمر 30 يوم، سحب الدم بعمر (28 و39) يوم من عمر الدجاج ، درست القابلية المستضدية للقاح بأستعمال الرحلان الكهربائي العمودي والتبصيم المناعي وحدد اربعة عشر نوع من متعددة الببتيدات وكانت اكثر استجابة مناعية بعد فحصها ضد امصال الدجاج الممنع بعمر 39 يوم حيث تراوحت اوزانها الجزيئية (167.8-114.5-83.4-78.2-53.3-44.1-38.8-36.4-28.2-20.5-18-14.9-13.9) كيلو دالتن وتم تحديد مستوى الانتزفيرون – غاما ($\text{INF}\gamma$)

والانترلوكين 4- (IL-4) في امصال الدجاج المحصن بأستعمال كيت الاليزا. اوضحت النتائج نوعين من الاستجابة المناعية الخلطية والخلوية ضد لقاح البروتيني للكيسات البيضبة لطفيلي الايميرية تنلا.

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