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Immune Responses to *Eimeria tenella* Sporozoite Protein as Vaccine to Broiler Against Coccidiosis

Suhair R. Al-Idreesi[1], Mahmoud Kweider[1] and Mahmad M. Katranji[2]
[1]Department of Animal Biology, Faculty of Sciences, Damascus University, Damascus, Syria
[2]Faculty of Veterinary Medicine, Al-Baath University, Homs, Syria

Abstract: The chicken was protected against coccidiosis induce by *Eimeria tenella* by using sporozoite protein that was injected in the neck subcutaneously with two doses (25 µg per chicken) at 3rd and 16th days of age, vaccinated birds were challenged at 30 days of age. The type of immune response to this vaccine was estimated during vaccination and after the challenge. Blood samples were collected at (7, 28 and 39th) day of age. The immunogenicity of vaccine was studied by using SDS-PAGE and Western blot. 11 polypeptides had been estimated more immunogenic after probing with immunized chicken serum at 39th days of age, their molecular weight are (149.5, 97, 72.9,67.8, 63,51, 38, 17, 13, 10.5 and 8) KD. Also, the levels of γ-IFN and IL-4 were estimated in the serum of immunized chickens by use ELISA kits. The results were demonstrated two types of immunity cellular and humoral responses against *E. tenella* sporozoite vaccine.

Key words: Vaccine, sporozoite, *Eimeria tenella*, western blot, IFN-γ, IL-4

INTRODUCTION

Apicomplexan protozoa of the genus *Eimeria* are a common cause of coccidiosis. Following ingestion of infective oocysts coccidial parasites undergo a complex life cycle ultimately malabsorption, body weight loss and in severe cases, death (Fitzgerald, 1980). An annual loss due to coccidiosis estimates in excess of 2 billion worldwide (Williams, 1998). So far, the only available prevention and control strategies against coccidiosis are anticoccidial drug and live oocyst vaccine (Ding *et al*., 2005; Jang *et al*., 2010). However, the increase of drug-resistant strains of *Eimeria tenella* raises the public concern of safety for the use of anticoccidial drugs in food- producing animals because of its chemical residues. Meanwhile, the use of a live oocyst vaccine has the possibility to cause risk of reverting bag to a pathogenic strain (Liu *et al*., 2013). The spozoite antigens are present on the surface and some of these molecules may be involved in recognition and penetration of host cell (Long, 1990). Sporozoites represent likely targets of protective immune response since in immune chickens they undergo a very restricted development or even fail to penetrate cells in the intestinal tract (Murray and Galuska, 1986). So, some study had been used a recombinant *E. tenella* sporozoite micronema antigen (EtMIC1) (Subramanian *et al*., 2008). Another study was used rhomboid-like protein of *E. tenella* as a subunit vaccine (Li *et al*., 2012). Subramanian *et al*. (2008) was found although the usefulness of (EtMIC1) as a potential candidate vaccine protozoan parasites have multiple life cycle stages. Finding a single target antigen that can elicit sterile immunity against coccidiosis is difficult. Therefore a mixture of antigens, which can act synergistically, may be trying to further reduce the oocyst output. So, other studies had been used total protein of sporozoite to *E. tenella* as a vaccine against coccidiosis in broiler which reduction in oocyst production (AL-Idreesi *et al*., 2013a; Badawy and Aggour, 2006; Kakhanis *et al*., 1991; Murray and Galuska, 1986).

Many studies had 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 (Lillehoj and Lillehoj, 2000; Lillehoj and Okamura, 2003).

Various cytokines are produced by macrophages following coccidial infection (Lillehoj and Trout, 1996). T-helper (Th). Th-secret γ-IFN and also tend to secret IL-2, while Th-2 cells secrete IL-4 and also tend to secret IL-5, IL-6 and IL-10. The cell type whose secretions dominates may help to determine the outcome of certain parasitic infections (Sher and Coffman, 1992). IFN production in chickens has been used as a measure of T-cell responses to coccidial antigens (Byrnes *et al*.,1993; Martin *et al*.,1994; Prowse and Palliter, 1989). So, the aim of current study is evaluating the type of immune responses when use sporozoite of *E. tenella* protein as vaccine from local strain against coccidiosis. This study has been estimated the γ-IFN and IL-4. Also, study the type of sporozoite antigenicity by Western blot technique to control this disease in Broiler.

Corresponding Author: Suhair R. Al-Idreesi, Department of Microbiology, College of Veterinary Medicine, Basrah University, Iraq

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 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 (Schamatz *et al.*,1984). Sporulated oocysts were stored in phosphate buffer saline (PBS PH = 7. 6) at 4°C until further use.

Preparation of sporozoite protein (Vaccine): About 2 mL (4×10^7) of purified sterilized oocysts 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 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 a 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 and some intact oocycts was then suspended in PBS. Purification of sporozoites was carried out by use either percoll gradient as described previously by Dulski and Turner (1987) or by using ion exchange chromatography (Schmatz *et al.*, 1984; Riggs and Perryman, 1987). Purified sporozoites were counted by using haemocytometer then stored in 1 ml PBS at -75°C until use. Solubilization of purified sporozoites were carried out by using 100 µL of lysate buffer (0.5% Nonidet P40, Tris-HCL 10 Mm, Aprotinen 0.1 U/mL, 1% TritonX-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 a source of protein (vaccine). The concentration of protein was determined by the method of Bradford assay (Wallach *et al.*, 1994).

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 on a 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 three separated floor pens and on litter composed of wood shaving to a depth 5 cm. Temperature in the floor pens was maintained 20-30°C. Extreme management was taken to avoid accidental exposure of chicks to coccidia during the immunization period. Also 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 in Table 1.

Table 1: Groups of experimental design

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

Immunization: A total number of 80 broiler chicks (Ross, 308) one-day old were divided as (Table 1) into 3 groups. G1 was immunized subcutaneously (S/C) in the neck with two doses: first dose at the 3rd day of age with 25 µg antigen (sporozoite protein) and a booster dose was given on 16th day of age with the same dose of protein. After two weeks of last immunization an oral inoculation with 10^4 of virulent *Eimeria tenella* sporulated oocysts for all groups except G3 which kept as unimmunized unchallenged control. Chicks in group G2 challenged only but didn't immunize.

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

Antigens characterization: *E. tenella* sporozoite antigens are identified by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE).

The extraction of *E. tenella* sporozoite protein (50 µg) per lane were lysed by boiling in reducing loading buffer {LB; 25% glycerol, 5% beta-Mercaptoethanol, 10% Sodium Dodecyl Sulfate (SDS), 0.01% Bromophenol blue in Tris/ HCl (PH = 6. 8)16 mM}. Then a separation was done by using (SDS-PAGE) on a 5-15 gradient gel. Polypeptide in the gel was transferred electrophoretically to nitrocellulose paper (BA85; 0.4 µM; Scheleiche and Schull, Inc.) In a Transblot transfer cell (Bio-Rad Laboratories). Electrophoresis were 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 {10 mM Tris-Hcl, 154 mM NaCl and 0.1% Tween -20} plus to 3% bovine serum albumin, for 24 h in 4°C.

The nitrocellulose paper was further washed 3 times, for 5 min. each time 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 1 h. Following washing, the nitrocellulose membrane was incubated for 1h in a 1:1000 dilution of {Rabbit anti-chicken IgG (H and L) conjugated to horse radish peroxidase (Invitrogen Company/California/USA), washed (3x5 min) in TBS-T buffer, finally incubated in OPD (Ortho Phenil domain) substrate solution for 15-20 min with rotating until the band had been appearing. The reaction was stopped by washing nitrocellulose stripes 2 times with D.W.

Production of interleukin-4 (IL-4) and interferon-γ (IFN-γ) induced by sporozoite protein in chicken: Serum chicken sample measured at 7, 28 and 39th days of age with ELISA kits (Life Science Inc. USCN) for the 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 µL of samples.

RESULTS

Antigens analysis: Polypeptides are obtained for analysis by the separation of sporozoite antigens by SDS-PAGE. There are 11 polypeptides were prominent stained strongly with coomassie blue. Their molecular weight were 10.9, 11.6, 13.9, 25.9, 37.5, 51.1, 77, 100, 107, 113 and 155 KD. Polypeptides also aggregated upon 155 KD which had a molecular weight greater than our used marker protein. Fig. 2a.

Reaction patterns of parasite-specific IgG (H-L) antibodies with sporozoite antigens differed when each of 3 immune sera were used as a probe in the immunobloting procedure. These differences were most obvious between molecular weight 8 and 149.5 KD Fig. 2b. Immunized serum of 39 day age of chicken consistently identified and reacted more intensely in sporozoite preparations than immunized serum at 28-7 day age of the chicken. These antigens with molecular weight 149.5, 97, 72.9,67.8, 63,51, 38, 17, 13, 10.5 and 8 KD were identified with immune serum post of challenge dose, by *E. tenella* parasite.

The immunized serum at 7 days age was reacted slightly when used as a probe. Five antigens had been reported with molecular weight 54, 38,13, 10.5 and 8 KD. Immune serum of 28 and 39 days age reacted with the same common antigens but slightly reacted.

All three immunized serum were reacted with sporozoite antigen of molecular weight 38,13, 10.5 and 8 KD.

Evaluation of IFN-γ and IL-4 production: After immunization with sporozoite 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 335.7 pg/100 µL at day 39, which was more than 56 fold increase as compared with control negative group (the data shown in Fig. 1).

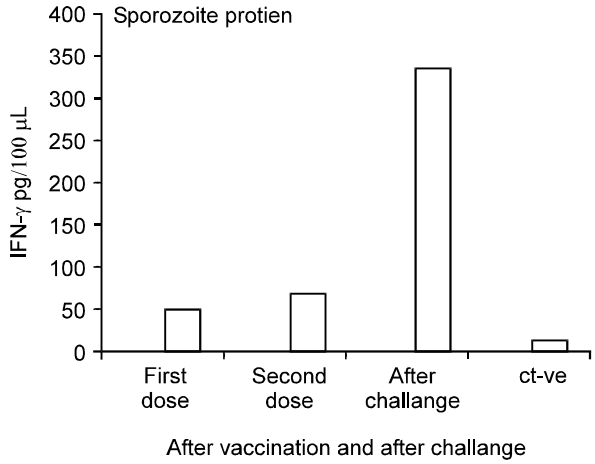

Fig. 1: Level of IFN-γ in serum of broiler after first vaccine dose (firs.dos), second dose (sec. dos) with sporozoite protein and after challenge (aft. chall) with 10^4 oocyst of *Eimeria tenella*

Also the results were demonstrated the level of IFN-γ increased gradually after first and second dose of sporozoite vaccine.

The average expression level of IL-4 was estimated to be 271 pg/100 µL at day 39, which appeared higher than control negative group which was 41.7 pg/100 µL (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 the second dose of vaccine.

DISCUSSION

Immunity to cocidiosis is of considerable academic interest because of the complicated life cycle of the organism and its obligate intracellular habitat. Principally in the intestine of the host (Rose, 1976). The development of chemo-resistant strains had lead the investigators to search for the development of vaccines (Akhtar *et al*., 2001). The protective immune responses in animals against coccidial infection is directed against the sporozoit stage, but it is generally accepted that the asexual stage produces the strongest stimulus for development of immunity (Long, 1990). So, current study use local Syrian isolate to prepare protein vaccine from the most important stage (sporozoite) in the life cycle of *E. tenella* parasite. Also, for a better understanding of strain variation is needed for any vaccine to give promising results against a local field strain of *Eimeria* (Anwar *et al*., 2008).

No oocyst were detected in the feces of control negative chickens throughout this study, demonstrating the

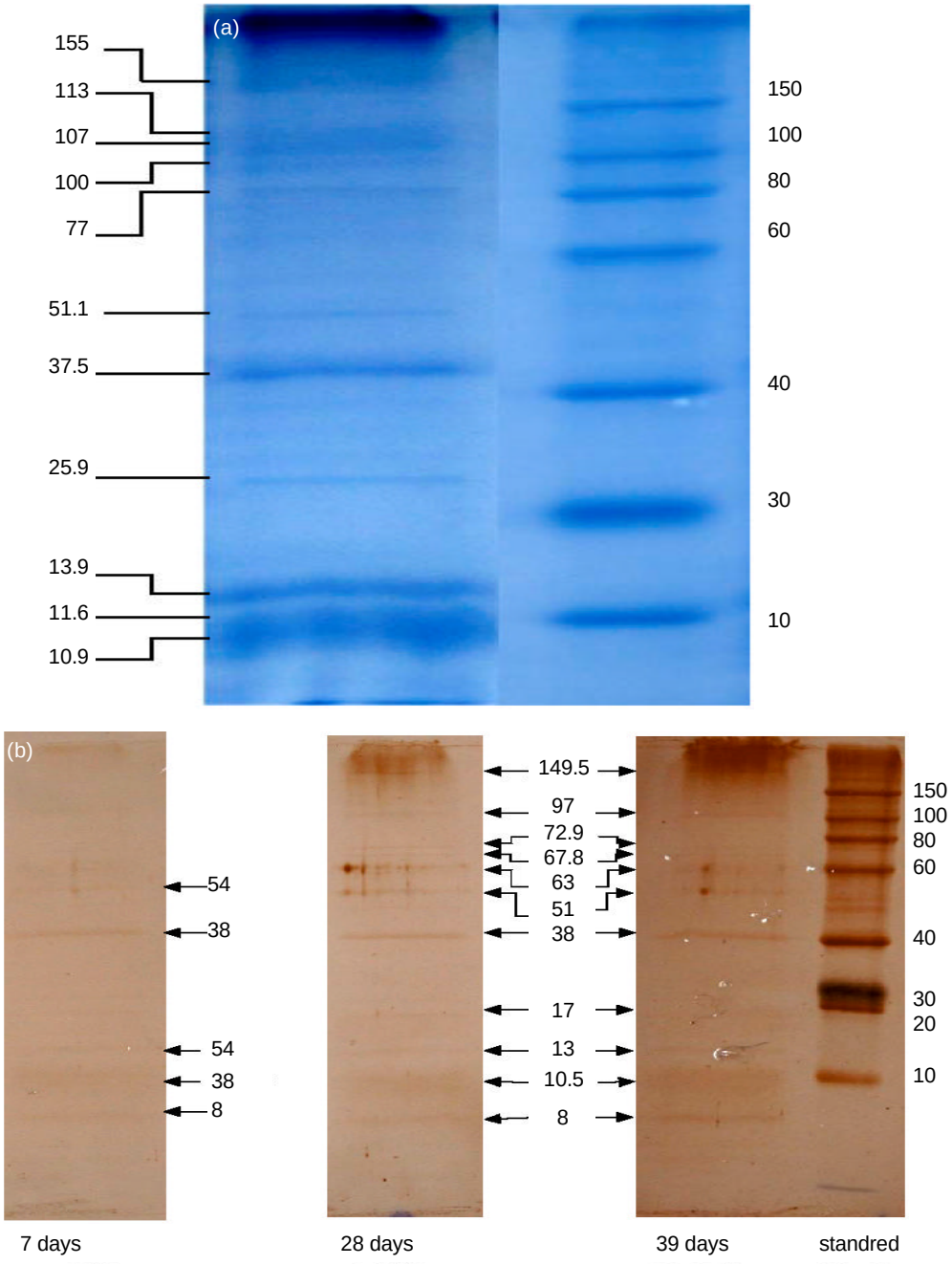

Fig. 2(a-b): Level of IL-4 in serum of broiler after first vaccine dose (firs. dos), second dose (sec. dos) with sporozoite protein and after challenge (aft. chall) with 10^4 oocyst of *Eimeria tenella*: (a) SDS-PAGE (5-15%) stained with Coomassie blue after electrophoretic separation of sporozoit proteins (Sz), (b) Immunoblot of proteins transferred from a 5-15% SDS-PAGE. Lanes are immunoblots of sporozoite extract preparations probed with immune serum (7 days): After first dose of vaccine (28 days): After second dose of vaccine and (39 days): After challenge with *E. tenella* oocysts

success of the procedure adopted to prevent contamination by extraneous coccidia. Further study of our used sporozoite vaccine demonstrated to significantly decreased of oocyst output and lesion scores as compared with control positive groups and provide chickens with protection rate around 99.2-99.5%. The body weight gain not affected (higher) in sporozoite immunized groups. Also, Anticoccidial Index (ACI) was

estimated which demonstrated that sporozoite protein was very effective (AL-Idreesi *et al*., 2013a).

In this study SDS-PAGE for sporozoites of *E. tenella* had been demonstrated many proteins (11 polypeptides) with Molecular Weight (MW) ranging from (10.9 to 155) KD, These polypeptides were (>10.9,11.6, 13.9,25.9,37.5,51.1,77,100,107,113,155) KD. A wisher (1986); Jenkins and Dame (1987) identified an impressive number of I^{125}-labeled immunodominant surface antigens as well as methionine-labeled and unlabeled cytoplasmic or inner membrane constituents of *E. acervulina* and *E. tenella*. Each species had from 32 to 45 prominent antigens ranging in size from 9 to 350 KD. Murray and Glausk (1986) were detected (11 polypeptides) from sporozoite of *E. tenella* protein when used SDS-PAGE their molecular weight are (235,175, 105,94, 82, 60, 50, 45, 28 and 23) KD. Another study speculated about the use of internal antigens to elicit protective immune response. Some of the antigens of great interest are located in the refractile body and have a molecular weight of 21 to 28 KD (Danforth and Augustine, 1989). These antigens are highly conserved among the *Eimeria* and apparently play important roles in the asexual development of the parasite (Augustine and Danforth, 1988).

There are different in molecular weight of polypeptides between the studies might be because of the differences in the condition of SDS-PAGE for each experiment and the differences in preparation of sporozoite proteins.

In immunoblotting study we observed differences in the identification of antigens and staining intensities among sera that were collected from chickens at (7, 28 and 39) days of age. After the first dose of currently used vaccine (four days from the first vaccine dose) we recognized five polypeptides; two of them reacted more intensely from the other with a molecular weight (38 and 8) KD while the number of proteins were reacted after the second dose of the vaccine (28 days of age) are the same number of proteins after challenge (39 days of age) but less intensity of reaction.

Wisher (1986) was obtained nine polypeptides when use western immune blotting to detect sporozoite of *E. tenella* antigen, the major molecular weight components detected were (14, 18, 23, 37, 42, 54,67, 73 and 113) KD.

This study was observed the most dominant polypeptide bands when use three immune sera as a probe in the immune blotting procedure were (38,13, 10.5 and 8) KD. While Paul *et al*. (1986) and Files *et al*. (1987) used a monoclonal antibody that inhibited penetration of *E. tenella* sporozoite *in vitro*, to identify a sporozoite surface antigen. In non reduced (SDS-PAGE), the protein migrated as a single band to an apparent molecular weight of 21 to 23 KD, while in reduced gels two bands were seen at 17 and 18 KD. Also, Brothers *et al*. (1988) were observed (by using neutralizing antibodies) 25 KD polypeptide on the surface of *E. tenella* sporozoites. This antigen consisted of two 17 and 8 KD polypeptides linked by a disulfide bond. The present study recognized 17 and 8 KD polypeptide bands. Radio-iodination of *E. tenella* sporozoites, to identify only the cell surface proteins, revealed five major proteins of (10,18,21,26 and 47) KD. (Lillehoj and Trout, 1993).

Al-Idreesi *et al*. (2013b) was observed 7 polypeptides appear more immunogenic after probed with chicken serum at 39 days of age (vaccine used was 125000 dead sporozoite of *E. tenella* parasite); Their molecular weight were (12.3, 13.68, 39, 59.5 and 77.3) KD. Another study had been reported 7 immunogenic surface sporozoite of *E. tenella* polypeptides (26,45,68,82,94,105 and 235) KD. Murry and Galuska (1986). While Augustian and Danforth (1985) were reported membrane proteins of 37 and 45 KD inhibited invasion of *E. tenella* but not *E. meleagrimitis*.

This study was revealing 38 and 51 KD polypeptides in the serum that collected after three times (first and second dose of vaccine and after challenge with *E. tenella* oocyst).

These differences or similarity between the present study and previous ones might be depend on the labeling reagent also cell solubilization has effects on antigens and the method used to determine immunodominant polypeptides.

In this study we used Rabbit anti-chicken IgG to stimulate antibody in the serum samples which were collected from the chickens in two times of vaccination and after the challenge, observed IgG was reach peak after challenge with oocyst of *E. tenella*.

Upon receiving the proper stimuli, beta-cells differentiate into plasma cells to secrete antibodies either in the absence (T-independent antigens) or in the presence (T-dependent antigens) of Th-cells. Regardless of the type of antigen, several types of immunoglobulin may be produced, including IgM, IgG or IgA. IgM is produced largest amount during a primary response, with more IgG bing produced during subsequent exposures to the stimulating antigen, responses are generally stronger and more rapid at this time also (Sher and Coffman, 1992)

Antibodies play role in the protective immunity against *Eimeria* (Wallach, 2010). Serum IgG titers peaked 13 to 17th days post infection with *E. tenella* parasite (Trees *et al*., 1985). Badawy and Aggour (2006) have been shown during their study on Sporozoit protein as vaccine when used Enzyme linked immune Sorbent assay, antibodies were detected on day 16th and reached a peak on day 40 post immunization. The antibodies in chicken convalescent sera taken at the time of peak IgG production were shown to be capable of providing excellent passive immunity against challenge infections in naïve chicks (Rose, 1974).

In the current study we agree with the last studies to the importance of antibodies in protective immunity against coccidioses and the immune serum on day 39 post immunization consistently identified and reacted more intensely with antigens in sporozoite preparation than immune serum at 7 and 28th days age of the chicken. While, Augustine and Danforth (1986) were demonstrated that antibody responses played minor role in protection against coccidiosis.

The results presented here provide further insight into the roles of cellular and humoral immunity during immunization first and second dose of sporozoite antigen and after challenge with *E. tenalla* oocysts. At the cellular level, we observed increased in serum levels of IFN-γ gradually after first and second dose of vaccine but increased drastically after challenge as compared with control negative group. Li *et al*. (2012) was noticed IFN-γ levels significantly higher in the serum samples when compared with those of the PBS group used rhomboid-like protein of *E. tenella* as a subunit vaccine in protective immunity against homologous challenge. Yuan *et al*. (2000) was observed during their study of kinetic differences in intestinal and systemic interferon-γ and antigen-specific antibodies in chickens experimentally infected with *E. Maxima*, serum IFN-γ production was significantly increased at (8-10) days post infection compared with uninfected control chickens at day (o) and decreased thereafter. But in the area of the intestine parasitized by *E. Maxima* IFN-γ reached maximum at day (4) post infection. These results clearly demonstrated that the intestinal cellular immune response, as measured by IFN-γ production, preceded the systematic appearance of IFN-γ . Cornelissen *et al*. (2009) was found during his study following *E. tenella*, the responses of IL-2, IL-4, IL-10, IL-18 and IFN-γ were not significantly elevated in the cecal on day 4th. But at 6^{th} days five cytokines (IL-2,IL-4, IL-10, IL-18.and IFN-γ) Showed significantly higher mRNA expression levels in the caecum as compared to non-infected animals. Laurent *et al*. (2001) showed that IFN-γ expression in the cecum and jejunum of White Leghorn chickens increased more than 200-fold at 7th days post primary infection with *E. tenella* and *E. Maxima*.

The immunized chickens were responses to the infection after the challenge and the intestinal lesions in coccidiosis are caused, in part, so in this study we observed high level of IFN-γ production after challenge as compared with control negative group. During infection, cytokines such as IFN-γ can stimulate inflammatory cells like macrophage to synthesize highly reactive free radicals, NO. These NO are not only toxic the invading parasite but also can damage the host tissue (Subramanian *et al*., 2008). Also Ovinyton *et al*. (1995) was observed the production of No^{-}_{2}+No^{-}_{3} and IFN-γ in serum increased during host response to infection of *Eimeria* and enhanced major histocompatibility complex class II antigen expression on macrophages (Lownthal *et al*. 1997). Indeed, a number of studies have shown that IFN-γ inhibits sporozoite replication in chicken cells both *in vitro* (Dimir-poisson *et al*., 1998; Lillehoj and Choi, 1998) and *in vivo* (Lillehoj and Choi, 1998).

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 vaccine and return to high level after challenge. While Cornelissen *et al*. (2009) was found following *E. acervulina* and *E. tenella* infection high IL-4 and IL-10 mRNA levels were also associated with reduced duodenal and cecal IFN-γ mRNA levels. Another study on analysis of chicken cytokine and chemokin gene expression following *E. acervulina* and *E. tenell* Infections, IL-4 and IL-13 mRNAs were decreased 25 to 2×10^{5}-fold after primary and secondary infection (Hong *et al*., 2006).

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. Also, IFN-γ stimulates B cells 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* (Tizard, 2000).

In this study the immune response to our experimental vaccine was demonstrated cellular and humoral protection. Many studies agree with these observations (Badawy and Aggour, 2006; Subramanian *et al*., 2008; Li *et al*., 2012). While other studies were found the cell-mediated immunity was playing a major role in protection from coccidia. (Bhogal *et al*., 1989; Marten *et al*.,1995; Dalloul and Lillehoj, 2005). Akhtar *et al*. (2001) and Wallach (2010) were shown the importance of antibodies to resist coccidioses.

Conclusion: This study observed two types of immune responses, humoral and cellular immune responses to sporozoite antigen which given more effective protection in broilers. Further studies are recommended to determine which bands of sporozoite antigens have the most immunogenic pattern to product this band by DNA level in large quantity to use this vaccine in the commercial market.

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