Parasitology Unit.,
Port-said Laboratory for Food Hygiene.

IMMUNE RESPONSES IN CHICKENS AGAINST *EIMERIA TENELLA* ANTIGEN

(With One Table and 2 Figures)

By

G.A. BADAWY and M.G. AGGOUR*

* Biotechnology Department, Animal Health Research Institute, Dokki.

(Received at 13/5/2006)

الاستجابة المناعية فى الدجاج ضد انتجن الأيميريا تينيلا

غريب عبد العزيز بدوى ، محمد جلال عجور

تـم تحصـين كتاكيت عند عمر ٢ ، ١٨ يوم بانتجن محضر من اسبوروزويتات حويصلات الكوكسيديا الأعورية (ايميريا تينيلا) باستخدام جرعة (٢٥ ميكروجرام / كتكوت) تحت الجلد فـى صورة مستحلب مع مساعد الفروينذز الكامل فأظهر حماية عند إجراء التحدى بجرعة مـن حويطلات الكوكسيديا المماثلة (10^4) عند عمر ٣٢ يوم. وكانت كفاءة التحصين أعلى عـن الكتاكيـت التي تم تحصينها بالانتجن أو مساعد الفروينذز الكامل كلا على إنفراد وتم مقارنـتهم بالمجموعـة الضـابطة الغـير محصـنة. وكان ذلك واضحاً فى إنخفاض عدد حويصـلات الكوكسيديا فى الدجاج المعدى (١,٢٧ ± ٠,١٦) × 10^7 /جرام من الزرق وفى إنخفـاض التغيرات الباثولوجية فى الأعورين (+١,٦) مع عدم وجود نفوق وكانت الاستجابة المناعية الخلوية الممثلة فى مؤشر هجرة الخلايا الليمفاوية كانت قد وصلت الى أعلى معدل لها ما بين يوم السادس عشر والثانى والثلاثون بعد التحصين. أما الأجسام المضادة بإستخدام اختـبار الالـيزا فتم الكشف عليها فى اليوم السادس عشر ووصلت لأعلى معدل عند اليوم الأربعون بعد التحصين.

SUMMARY

The chicken on 2nd and 18th day of age were immunized subcutaneously with *Eimeria tenella* sporozoite antigen (25 ug per chicken) emulsified in freund's complete adjuvant (FCA) showed protection against challenge with homologous sporulated oocyst (10^4) at 32 day of age. The immunization efficacy was greater than those immunized with the antigen or FCA alone as compared with the unimmunized control. This was evident by a reduced number of oocyst production (1.27±0.16) × 10^7 per gram feces, lower mean lesion scores (+1.6) with no mortalities. Lymphocyte migration (LM) index represented the cell mediated

immune response (CMIR) indicated that its peak reached between day 10 and day 32 post-immunization in chickens immunized with *Eimeria tenella* sporozoite antigen. ELISA antibodies were detected on day 16 and reached peak on day 40 post-immunization.

Key words: *Coccidiosis, Eimeria tenella, immunology, chicken*

INTRODUCTION

Caecal coccidiosis, a disease caused by a protozoan parasite *Eimeria tenella,* represents a severe problem for the poultry industry throughout the world due to losses from mortality. Also, growth and performance of the bird are heavily impaired. Today, the disease is controlled by preventive medication using polyether ionophores or chemical agents as anticoccidial drugs. In the past few years, prophylactic chemotherapy has lost some of its efficiency due to the appearance of resistant strains of the parasite (Chapman, 1993). This led us to a search for new approaches to control coccidiosis involving immunological, biotechnological and genetical methods. Of these, immunological approach is considering more importance. Various types of *Eimeria tenella* derived antigens as sporulated oocysts, sporozoites, merozoites antigens have been tried as vaccinal lines with various degrees of success (Wisher, 1986; Chapman, 1988; Sutton *et al.,* 1989 and Rhalem *et al.,* 1993). Of these, sporozoites are potential candidates as vaccinal antigen as they are the first developmental stage which comes in direct contact with the host immune system (Constantinoiu *et al.,* 2003). Keeping this in view, the present study was planned with an aim to immunize chickens against *E. tenella* using sporozoite antigen and to monitor the antibodies and cellular immune responses produced by this antigen.

MATERIALS and METHODS

Experimental chicks

One day old broiler chicks (Hubbard) were fed on non-medicated broiler diet, raised in wire floored cages under coccidial free conditions and without vaccine administration against any disease of poultry. Their faeces were examined periodically by the flotation technique for the presence of coccidial oocysts. Prior to use in experiment, one or two 3-5 weeks old chicks from each group were killed and the gastrointestinal tract and contents were examined to confirm the absence of coccidia.

Eimeria tenella strain:-

A line of *E. tenella* was established with single oocysts from a field sample as post-mortem case brought to department of poultry diseases, Animal Health Research Institute. The infected caecum was examined grossly and microscopically to identify the species of coccidia. Identification was based on the appearance of characteristic schizonts and gametocytes in fresh smear of caecal mucosa (Davies *et al.*, 1963). For propagation of *E. tenella* oocysts, 3 weeks old broiler chickens (Hubbard) were infected with 5000 sporulated oocysts. The faeces were collected daily in 2.5% potassium dichromate solution from the 5^{th}- day post inoculation and the collection was continued for another 4 days. *E. tenella* was maintained by passage through coccidia-free chicken. The unsporulated oocysts were obtained on day 7 post-infection from the caecal contents, purified and allowed to sporulate using standard procedures (Bontemps and Yvore, 1974).

Harvesting and counting of oocysts

The oocysts were purified by salt flotation technique, sporulated and sterilized by hypochlorite treatment. The counting of the oocysts was carried out by using the McMaster technique (Davies, 1973 and Long *et al.*, 1976). Purified oocysts were suspended in 0.1 M PB (ph7.6) at the concentration of 5x107 oocyst/ml.

Collection of sporozoites

2ml from the suspension of sporulated oocysts ($5x10^7$) was ground at 500 rpm for 10 minutes at 4°C in a tissue homogenizer. Following centrifugation (1000 rpm for 10 minutes), the supernatant was discarded and excystation of sporozoites from the revealed sporocysts in the pellet was carried out as described by (Davies *et al.*, 1963). The freshly obtained sporozoites were purified from the excystation debris by using a DE-52 cellulose anion exchanger as described by (Schmatz *et al.*, 1984).

Assessment of the viability of sporozoites within the sporulated oocysts

Maturation and viability of sporozoites were assessed by (excystation test) using an excystation medium composed of 0.25% trypsin in 1% sodium taurocholate in tris-maleate-NaOH buffer at 7.4. The excystation percentage could be estimated after incubation of mechanically released purified sporozoites for 1-2 hours at 4°C (El Kasaby, 1973). Culture with an excystation percentage between 80-95 % were used for experimental work, while less than 80 % was stored for maintenance culture.

Preparation of sporozoites antigen

Sporozoites $(10)^9$ were washed three times in 0.1 M PBS (pH 7.6) containing 1mM (PMSF) phenyl methyl sulfonyl fluoride (Sigma). They were then sonicated with Vir Sonic 475 (Virtis, USA) at a power setting of 3x and 30% duty cycle for 10 minutes. Sonication was performed on ice and sample was examined microscopically for disruption. The sonicated material was then centrifuged at 5000 rpm for an hour at 4°C and the supernatant used as soluble antigen for further experiments.

Experiment design

A total number of 100 one-day old broiler chicks (Hubbard) were divided into four groups and immunized as follows:

Group A: 25 chicks were vaccinated subcutaneously (s/c) on the breast region at 2 days of age with 25 µg antigen emulsified in Freund' s Complete Adjuvant (FCA- Sigma, St Louis USA) and a booster dose of 25 µg antigen alone was given on 18 days of age.

Group B: 25 chicks were vaccinated s/c with 25 µg antigen alone at 2 and 18 days of age.

Group C: 25 chicks were inoculated with FCA alone on 2 and 18 days of age.

Group D: 25 chicks were kept as unimmunized control.

Extreme care was taken to avoid accidental exposure of chicks to coccidia during immunization period and faeces from each group were periodically examined for the absence of *Eimeria* oocysts.

All the chicks were challenged at 32 days of age with 10^4 *E. tenella* sporulated oocysts per bird and the surveillance for coccidian oocysts was continued until the fifth day post - challenge.

Lesion scoring and faecal oocysts count

On sixth day post- challenge, 10 chicks from each group were killed and lesion scores assessed by the method of Johnson and Reid (1970). Briefly, 0 = no gross lesions; +2 = more numerous lesions with somewhat thickened cecal walls; +3 = greatly thickened cecal walls with large amounts of blood or cecal cores; +4 = greatly distended cecal walls with blood or large caseous cores. Also, the faeces from each group were collected separately from the fifth to the ninth day post- challenge and oocysts counted per 1 gm of faeces from each group using Mc Master's counting technique.

Immunological studies

Blood samples were obtained from chickens by cardiac puncture, allowed to clot for 4 hours and centrifuged (10 min, 2000 rpm). The sera were aliquoted in 1 ml vial and kept at -20^{0}C.

The heprinized whole blood and serum were collected from 3 chicks selected randomly from each group on 4, 8, 16, 32, and 40 days post-immunization. After collecting blood from chicks, they were marked with leg bands so that they were not reused for blood collection. However, all the chicks were challenged at 32 days of age.

Lymphocytes migrating inhibition test

This was performed according to Chbabra and Goel (1981) and Garg *et al.*, (1999). 5 ml of heprinized blood was layered over 3 ml lymphoprep in 15 ml centrifuge tubes and centrifuged at 400 xg for 25 minutes. The leucocytes at the interface of plasma and lymphoprep were collected with Pastuer pipette and washed twice with culture medium (RPMI 640 containing foetal calf serum, pH 7.1). The pellet was finally suspended in culture medium at concentration of $2x10^7$ to $5x10^7$ cells per ml. Microcapillaries were filled with this cell suspension, plugged at one end with plasticin and centrifuged at 200xg for 3 minutes. The capillaries were cut at the liquid cell interface and the stubs were anchored over migration surface of the chamber wells with the help of silicon grease applied at the bottom. A dose of 45 ug antigen / ml of RPMI-1640 (minimum toxic dose) was used to change the wells of LMI plate and area of lymphocyte migration was noted after incubating the plate for 18 hours at 37^{0}C.

Lymphocyte migration (LM) index was calculated as follows:

$$\text{LM index} = \frac{\text{Average of Lymphocyte migration in presence of antigen}}{\text{Average of Lymphocyte migration in absence of antigen}}$$

Enzyme linked immunosorbant assay (ELISA)

Flat bottomed 96-well microtitre plates (Nunc) were coated with sporozoites antigen according to Onaga *et al.*, (1986) and Garg *et al.*, (1999). Distilled water rinsed wells of microtitre plates were coated for 3 hours at 37°c with 50 µl of 1:20 dilution of *E. tenella* sporozoites antigen (80 µg / L). The coated plates were further incubated at 4°C for 18 hours. The coated plates were washed once with 0.1 MPBS containing 0.05 % (v/v) Tween 80 (pH 7.6) and air dried. Sera were diluted 1/20 in PBS containing 1% bovine serum albumen (BSA). The

diluted sera were incubated on *E. tenella* sporozoites antigen coated microtitre wells (4 µg antigen/ well) of the microtitre plate at 37°C for an hour. Serum was removed and wells were washed three times in washing solution. 50 µl of anti-chicken IgG (Sigma, Chemical Co.), diluted 1/1000 and conjugated with horse-radish peroxidase were then added and incubated at 37°C for an hour. Once again the plate wells were washed four times with washing solution. Antibody levels expressed as mean absorbance values. Absorbance values were read at 405 nm in an automatic microelisa reader (MR 610 autoreader, Dynatech Laboratories).

RESULTS

The immunological parameters used for determination of the immunizing efficacy of *E. tenella* sporozoite antigen were: mean oocyst production, mean lesion scores and mortality percentage. These parameters were shown in Table (1) after homologous sporulation oocyst challenge in different groups of chicken. The chickens were immunized with *E. tenella* sporozoite antigen emulsified in FCA showed reduction in oocyst output (1.27±0.16) × 10^7 oocyst per gram feces (OPG) as compared with unimmunized control (3.82±0.37). The same immunized group had mean lesion scores of (+1.60) as compared with the unimmunized control (+3.80). On the other hand, chickens immunized with antigen alone or FCA alone showed also reduction in oocyst output than unimmunized control group (2.98±0.22) and (3.17±0.46) with mean cecal lesion score of (+2.40) and (+3.50) respectively. There is no mortality in chickens immunized with antigen plus FCA which reached highest percentage (35%) in unimmunized control.

Lymphocyte migration (LM) index represented the cell mediated immune response (CMIR) in the immunized and control groups was calculated Fig. (1). Group A show a significant (CMIR) which ranged between 0.491 (±0.034) P≤ 0.05 in day 16 post immunization (PI) and 0.463 (±0.076) P≤ 0.05 in day 32 PI. In contrast, group B and group C (CMIR) was noticed at day 16 (PI), 0.543 (±0.019) P≤ 0.05 and 0.621 (±0.027) P≤ 0.05 respectively. On the other hand (CMIR) in the unimmunized control group (D), CMIR along, the post immunization period had no significant values.

The ELISA mean absorbance values at 405 nm of chicken sera in different immunized and control groups are shown in Fig. (2). In groups

A and B (chickens immunized with antigen given with adjuvant or alone) showed a significant increase in mean absorbance values at day 16 PI reaching peak at day 40 PI (0.389±0.029 and (0.341±0.007 P≤ 0.05) in both groups respectively. The unimmunized control group showed low mean absorbance values ranged from 0.111±0.014 (day 4 PI) to 0.158±0.009 (day 40 PI).

The above results indicated that immunization of chickens with *E. tenella* sporozoite antigen emulsified in FCA had immunizing efficacy than antigen or FAC alone.

Table 1: Immunological parameters in different immunized groups of chickens with *E. tenella* sporozoite antigen and unimmunized control group after challenged with homologous sporulated oocyst (10^4)

Groups	Treatment	Oocyst output ($\times 10^7$)	Mean cecal lesion scores	Mortality percentage
A	Antigen + *FCA	**1.27±0.16	+1.60	0
B	Antigen alone	2.98±0.22	+2.40	5
C	FCA alone	3.17±0.46	+3.50	10
D	Unimmunized control	3.82±0.37	+3.80	35

*FCA : Freund's Complete Adjuvant. ** = mean ±S.E., n=10
N. B: - Number of chickens in each treatment group=20

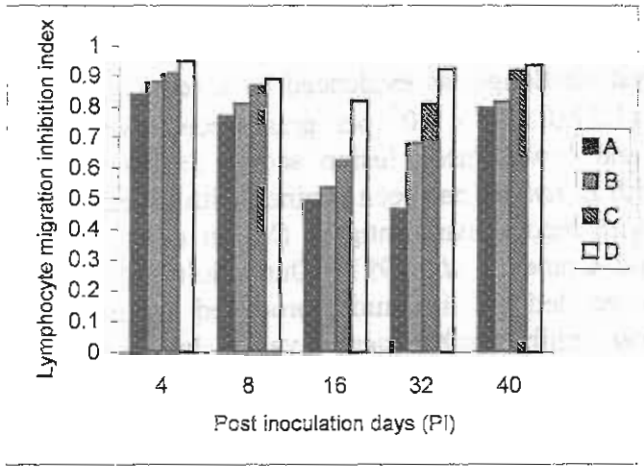

Fig. 1: Mean lymphocyte migration inhibition indices of chicken blood in different immunized groups with *E. tenella* sporozoite antigen and unimmunized control group.

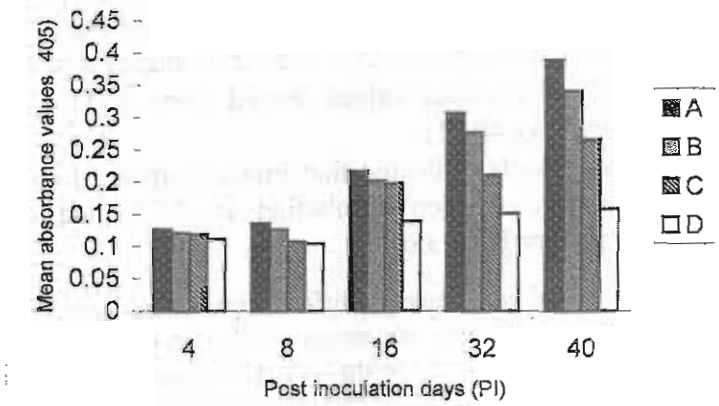

Fig. 2: ELISA means absorbance values of chicken sera in different immunized groups with *E. tenella* sporozoite antigen and unimmunized control group.

DISCUSSION

The criteria for judgment of the immunizing efficacy of *E. tenella* sporozoite antigen were mean oocyst output, mean cecal lesion scores and mortality percentage.

In the present study we found that chickens immunized with the sporozoite antigen of *E. tenella* emulsified in FCA showed protection against oocyst challenge, as evidenced by a reduced number of oocyst production (1.27±0.16) × 10^7 per gram feces. Also, we noticed no mortalities and lower mean lesion scores (+1.6). Protection against challenge with *E. tenella* has been attained with killed sporozoites (Rose, 1982) and with recombinant antigens (Miller *et al.*, 1988; Danforth *et al.*, 1989 and Crane *et al.*, 1991). Our results agree with Augustine (2001) who recoded that immunity produced against cecal species of avian *Eimeria*, inhibits subsequent invasion by homologous challenge species.

Cecal lesions evaluated by the method of Johnson and Reid (1970) rated on a scale of 0 (no pathological changes) to +4 (most severe pathological lesions) on the basis of subjective observation. Lesion scores of less than +2.0 represented cecal coccidiosis which would not have serious effect on the broiler performance. Thus, in our study the

mean lesion score of +1.6 in chickens immunized with 25 ug antigen emulsified in FCA represents a substantial degree of protection. Karkhanis *et al.*, (1991) were able to induce significant reduction in mean cecal lesion scores (less than +2.0) with less than 10 ug alum precipitated sporozoite antigen intramuscularly in the thigh on 2nd , 9th and 16th day of age of chickens. The difference could be due to the repeated inoculation with in the latter case. Dealing with the mortality percentage, it seems that no mortalities occurred in chickens immunized with antigen emulsified in FCA which reached 35% in unimmunized control group.

It is important to study the immunological response of chicken to infections caused by *Eimeria spp.* because control of this disease is a specific problem in conditions of intensive poultry production. The details of the protective mechanisms which are activated in the course of a coccidial infection have still not been satisfactorily explained, but it is known that cell mediated immunity plays a dominant role in defending the host from this agent. Intra-epithelial lymphocytes (IEL) play a very important role in the local defense of mucosa from coccidial invasion (Ilic *et al.*, 2003). Determination of cell mediated immune response (CMIR) of chickens immunized with *E. tenella* sporozoite antigen will be adopted by using ELISA and lymphocyte migration inhibition test. The results of cell mediated immune response which represented by the lymphocyte migration inhibition test using blood lymphocyte indicated that the peak of CMIR reached between day 16 and day 32 post immunization in chicken immunized with *E. tenella* sporozoite antigen. These results to somewhat agree with those obtained by Bumstead *et al.* (1995) who recorded peak CMIR between 14 and 21 days post infection by performing lymphocyte proliferation assay after oral immunization of bird with 100 sporulated *E. tenella* oocysts at 4 weeks of age and challenging the birds with 10000 sporulated *E. tenella* oocysts at 35 days post immunization. The difference may be attributed to route of administration and type of antigen (sporulated oocyst or sporozoite).

Rose and Mockett, (1983) recoded that ELISA has proved to be the most convenient and sensitive technique with regard to ease of preparation, evaluation time and quantities of antigen and test samples required for detection of antibody responses to *Eimeria* species. However the specific serum ELISA antibodies are detected shortly after one week of infection (Lillehoj and Ruff, 1987). Our results revealed that ELISA antibodies were detected on day 16 and reached peak on day 40 post immunization (group A). These were substantiate the findings of

Onaga *et al.* (1989) who found that antibodies against *E. tenella* are produced for ≥ 22 days and protection was conferred on most of the chickens by 38 days post immunization.

REFERENCES

Augustine, P.C. (2001): Sporozoite interactions and invasion by apicomlexan parasites of the genus *Eimeria.* Internat. J. Parasit., 31(1): 1-8.

Bontemps, M. and Yvore, P. (1974): Techniques de purification des suspensions de sporozoites d' *Eimeria* sur colonnes de fibres synthstiques. Ann. Rech. Vet., 5: 109-113.

Bumstead, J.M.; Bumstead, N.; Rothwell, L. and Tomley, F.M. (1995): Comparison of immune responses in bred lines of chickens to *Eimeria maxima* and *Eimeria tenella.* Parasitol., 111, 143-151.

Chapman, H.D. (1988): Strategies for the control of coccidiosis in chickens. Proc.5th European Multi Colloquium Parasitology, Budapest, Hungary, 4-7 September 1988.

Chapman, H.D. (1993): Resistance to anticoccidial drugs in fowl. Parasitolo. Today, 9: 159-162.

Chbabra, P.C. and Goel, M.C. (1981): Immunological responses of chickens to *Mycoplasma gallisepticum* infection. Avian Dis., 25: 279-282.

Constantinoiu , C.C; Lillehoj, H.s.; Matsubayashi, M.; Hosoda, y.; Tani, H.; Matsuda, H.; Sasai, K. and Baba, E. (2003) : Analysis of cross – reactivity of five new chicken monoclonal antibodies which recognize the apical complex of *Eimeria* using confocal laser immunofluorescence assay. Vet. Parasit., 118 (1-2): 29 – 35.

Crane, M.S.; Gaggin, B.; Pellegrane, R.M.; Ravino, O.J.; Lange, C.; Karkkanss, Y.D.; Kirk, K.E. and Chakraborty, P.R. (1991): Cross protection against four species of chicken coccidia with a single recombinant antigen. Infect. Immun., 59 : 1271-1277.

Danforth, H.D.; Augustine, P.C.; Ruff, M.D.; McCandliss, R.; Strawbery, R.L. and Likel, M. (1989): Genetically engineered antigen confers partial protection against avian coccidiosis. Poult. Sci., 68 : 1643-1652.

Davies, S.F.; Joyner, L.P and Keendall, S.B (1963): Coccidiosis. Oliver and Boyd, Edinburgh and London.

Davies, I.R. (1973): Techniques , in: D.M. Hammand and P.I. Long (eds) The *Eimeria, Isospora, Tosxoplasma* and related genera, pp. 411-450 (USA, Boltimore, University. Park Press).

El Kasaby, A.M. (1973): The mechanism of excystation of coccidia in poultry. Cited from Ph. D. Thesis, Faculty of Science, Univ. of London.

Garg, R.; Banerjee, D. and Gupta, S. (1999): Immune responses in chickens against *Eimeria tenella* sporozoites antigen. Vet. Parasit., 81: 1-10.

Ilic, T.; Knezevic, M.; Aleksic, K. and Dimitrijevic, S. (2003): Immunological response of chicken to coccidial infection. Veterinarski-Glasnik; 57 (7-8): 501-504.

Johnson,J. and Reid, N.M. (1970): Anticoccidial drugs. Lesion scoring technique in battery and floor pen experiments with chickens. Exp. Parasitol., 28: 30-36.

Karkhanis, Y.D.; Noltstadt, K.A.; Bhogal, M.S.; Ravino, C.; Pallergrino, R.; Crane, M.S.; Murray, P.K. and Tuner, M.J. (1991): Purification and characterization of a protective antigen from *Eimeria tenella*. Infec. Immun. 59: 983-989.

Lillehoj, H.S. and Ruff, M.D. (1987): Conjugation of disease susceptibility and subclass – specific antibody response in SC and FP chickens experimentally inoculated with *Eimeria tenella*, *Eimeria acervulina* and *Eimeria maxima*. Avian Dis., 31: 112-119.

Long, P.L; Joyner, L.P; Millard, B.J. and Norton, C.C. (1976): A guide to laboratory techniques used in the study and diagnosis of avian coccidiosis. Folia Veterinaria Latina, 6: 201-217.

Miller, G.A.; Bhogal, B.S.; Anderson, A.C.; Jersee, E.J.; Strausberg, S. and Strausberg, R. (1988): Application of a novel recombinant antigen in a vaccine to protect chickens from coccidiosis. Prog. Clin. Biol. Res., 307: 117-130.

Onaga, H.; Saeki, H.; Hoshi, S. and Veda, S. (1986): An ELISA for serodiagnosis of coccidiosis in chickens. Use of a single serum dilution. Avian Dis., 30: 658-661.

Onaga, H.; Togo, M.; Kudo, Y.; Motohashi, T. and Ishi, T. (1989): The use of enzyme linked immunosorbant assay for estimation of the immune status of chickens artificially immunized against coccidiosis. Vet. Parasitol., 33: 199-205.

Rhalem, A.; Sahibi, H.; Dakkak, A.; Loument, F.; Kazanji, F.; Yvore, P. and Perry, P. (1993): Protective oral immunization of chickens against *Eimeria tenella* with sporozoite surface antigen. Vet. Immunol. Immunopath., 39: 327-340.

Rose, M.E. (1982): Host immune responses. In: Long, P.L. (Ed.). The biology of the coccidian, University Park Press, Baltimore, Maryland. pp. 329-371.

Rose, M.E. and Mockett, A.P. (1983): Antibodies to coccidia detection by ELISA. Parasit. Immunol., 5 : 479- 489.

Schmatz, D.M.; Crane, M.S. and Murray, P.K. (1984): Purification of *Eimeria* sporozoites by DE-52 avian exchange Chromatography. J. Protozool., 31:181-183.

Sutton, C.A.; Shirley, M.W. and Wisher, M.W. (1989): Characterization of coccidial proteins by two-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis. Parasitol., 99: 175-187.

Wisher, M.H. (1986): Identification of the sporozoite antigens of *Eimeria tenella.* Mol. Biochem. Parasitol., 21: 7-15.