Immunoglobulin producing cells in chickens immunized with *Eimeria tenella* gametocyte antigen vaccines

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ABSTRACT: The present paper reports on the IgA, IgG and IgM antibodies secreting cells (ASC) in the spleen of chickens vaccinated with *E. tenella* (local isolates) gametocyte vaccine(s) by using Enzyme Linked Immunospot (ELISPOT) assay. Irrespective of the vaccine used, the number of IgG antibody secreting cells (ASC) in the spleen of orally vaccinated chickens was higher than the number of IgA and IgM ASC. Maximum numbers of IgG, IgA and IgM ASC were found in chickens vaccinated with sonicated gametocyte formalin inactivated vaccine (Group III) followed by formalin inactivated gametocytes (Group II) and gametocytes (Group I). The number of ASC (IgG, IgA and IgM) per 10^4 cells in spleen was significantly higher ($P < 0.05$) in Group III as compared to Group II and Group I. Results of the challenge experiments revealed maximum protection against mixed species of the genus *Eimeria* in Group III (77.8%) followed by Group II (55.6%) and Group I (52.0%). Lesion scoring was directly proportional to the per cent mortality and oocysts per gram of droppings but inversely proportional to the per cent protection. Based on the results, it was assumed that the spleen in chickens is one of the major sources of cells producing IgA, IgG and IgM antibodies.

Keywords: *Eimeria tenella*; gametocytes; antibodies secreting cells; ELISPOT

Chickens infected with *Eimeria* species produce parasite-specific antibodies that are present in the systemic circulation and mucosal secretions (Lillehoj and Trout, 1996). Like in mammals, three principal classes of antibodies including IgM, IgA and IgG (IgY) are known in chickens (Lebacq-Verheyden et al., 1972). Upon exposure to *Eimeria* species, chickens produce all three classes of antibodies. The cells expressing *E. tenella* specific IgG have been detected in the lamina propria of the caeca and in the red pulp of the chicken spleen (Vervelde et al., 1992).The IgM secreting plasma cells in the lamina propria of the villi upon *E. tenella* infection have been detected (Jeurissen et al., 1989). An elevated level of IgA has been found in a tissue parasitized with *E. tenella* (Girard et al., 1997). The present paper reports on IgM, IgA and IgG antibody secreting cells in the spleen of chickens immunized with egg propagated *E. tenella* gametocyte vaccines.

MATERIAL AND METHODS

Ex-sporocystation

Sporulated oocysts of *E. tenella* (local isolates) maintained in Immunoparasitology Laboratory, University of Agriculture, Faisalabad, Pakistan were processed for excystation followed by ex-sporocystation to release sporozoites following the method of Speer et al. (1973). Briefly, purified sporulated oocysts were treated with 2.5% sodium hypochlorite for 20 min followed by continuous stirring in a vessel containing sterilized glass beads (425–600 µm diameter) for 25 min on a magnetic stirrer. The excysted material was centrifuged (310 × g for 10 min), the supernatant was discarded to remove sodium hypochlorite and the sediment was given three washings with phosphate-buffered saline (PBS). The washed sporocysts were suspended in excystation fluid (0.25 g trypsin, 4.0 g tauro-

deoxycholic acid, and 0.094 g magnesium chloride brought to 100 ml volume with HBSS at pH 7.8–8.0) followed by homogenization for 1 × 7 min (cycled on/off for 30 min on ice) at 40°C and 5% CO_2. The excysted sporozoites were obtained by centrifugation (310 × g for 10 min) and their concentration was adjusted to 1.8×10^3–2×10^3 per 0.1 ml in PBS and stored at 4°C for further use.

Egg propagated gametocytes

Five hundred chicken embryos (9-day-old) procured from a local hatchery were maintained at 39°C and 70% humidity in an incubator. Candling was performed to know the status of the embryo. At 12 days of age, 0.1 ml suspension of sporozoites were inoculated into the chorioallantoic membrane along with penicillin and streptomycin using a sterile 25-guage needle and placed in an incubator at 39°C and 70% humidity for 5–7 days. On day 5–7 post-inoculation, the chorioallantoic fluid was collected from the dead embryos and gametocytes (macro and micro) were harvested (Akhtar et al., 2002a). Gametocytes were purified by centrifugation (14 500 × g for 5 min) and washed twice with PBS. The protein concentration of egg propagated gametocyte antigen was measured (Bradford, 1976) and adjusted to 500 µg/0.2 ml with PBS. The following gametocyte vaccine(s) were prepared:

Vaccine I – gametocytes

Vaccine II – gametocytes inactivated with formalin (37% formaldehyde) making the final concentration as 3% v/v (Fue and Lee, 1976).

Vaccine III – gametocytes sonicated for 1 × 5 min by using an ultrasonic homogenizer (Nissei, Model US 330, Japan) in a jacketed vessel at 4–8°C. The homogenate was centrifuged (250 × g for 15 min). Soluble material above the pellet (supernatant) collected was inactivated with 3% formalin (Fue and Lee, 1976).

Experimental chickens

Four hundred one-day-old broiler chicks (Hubbard) procured from a local market were reared (SPF environment; coccidian free) at an experimental station, Department of Veterinary Parasitology, University of Agriculture in Faisalabad, Pakistan, under standard management conditions. The water and feed were offered *ad libitum*. On the 5th day, chickens were divided into four equal groups (I, II, III and IV). Vaccine I, Vaccine II and Vaccine III were given orally to group I, II and III, respectively; each vaccine dose contained 500 µg of protein per 0.2 ml (Ayaz, 2003). Chickens in the control group (Group IV) were given normal saline (0.2 ml orally).

On day 15 post vaccination (pv), the spleen was collected from each group (45 chickens) to be used in Enzyme Linked Immunospot assay. The remaining chickens in each group (45 chickens) were challenged with 70 000 sporulated oocysts (mixed species of the genus *Eimeria*, mainly *E. tenella*, *E. acervulina*, *E. maxima* and *E. necatrix*; local isolates) on day 16 pv (Hein, 1970). Faecal examination was conducted daily and the number of oocysts per gram of droppings was determined by McMaster counting technique (Ryley et al., 1976). Per cent protection was calculated from the survived chickens in each group.

On day 21 pv, survived chickens in each group were scarified for lesion scoring (Johnson and Reid, 1970). Lesion scorings of the dead chickens were also conducted. Lesions on intestines and caeca were enumerated and scored from 0 to 4.

Enzyme Linked Immunospot (ELISPOT) assay

ELISPOT assay was used to detect the IgA, IgG and IgM specific antibody secreting cells in the spleen of chickens vaccinated with gametocyte vaccine(s) (Ayaz, 2003). For this purpose, each spleen was crushed over by pressing on the fine mesh in Petri dishes containing RPMI-1640 (Sigma, Aldrich Cheme GmbH, Germany). The suspension was then passed through a nylon cell strainer (70 µm; Becton, Dickinson, Lincoln Park, NJ). The filtrate was centrifuged (250 × g for 10 min at 4°C) and the sediment was collected. Lysis buffer (1 ml/spleen) was added for erythrocyte lysis and placed on ice for 2 minutes. The suspension was passed through the cell strainer again and centrifuged (250 × g for 10 min at 4°C) to collect the sediment. The cell suspension (10 µl) was mixed with the same amount of trypan blue (Sigma, Aldrich Cheme Gmbh, Germany) and the number of cells was counted in a haemocytometer. Their concentration was adjusted to 10^6 cells/100 µl with RPMI-1640.

Test procedure

Coating with Ig-specific primary antibody. Nitrocellulose-baked microtitre plates (96 wells;

Millipore multiscreen MAHA N4550) were used in the ELISPOT assay. Individual wells of the plate were filled with 100 µl of Goat Anti-chick Ig (H+L)-UNLB (primary antibody) at a final concentration of 2 µg/ml and were allowed to stand overnight at 4°C in a humid chamber. Unadsorbed antibodies were removed by three successive washings with PBS. Wells were immediately filled with 100 µl RPMI-1640 to saturate the remaining binding sites and incubated at 37°C for 2 hours. The medium was discarded and the plates were dried with absorbent paper.

Incubation of Ig-secreting cells. A 100 µl cell suspension containing 10^6 cells was dispended into each well in duplicate and they were incubated undisturbed at 37°C for 4 hours. The plates were rinsed twice with PBS by manual shaking and then three times by immersion in PBS containing 0.05% Tween 20 (PBST) for 2–3 minutes. The wash buffer was removed from the plates and the outer surfaces of the plates were dried carefully.

Addition of labelled secondary Ig-specific antibody. A 100 µl of PBST containing Goat Anti-chick IgA-AP (1 000-fold dilution), Goat Anti-chick IgG-AP (1 000-fold dilution) and Goat Anti-chick IgM-AP (1 000-fold dilution) were added to each well and the plates were incubated at 4°C overnight. The plates were then rinsed three times by immersion in PBST and dried. Each well was then filled with 100 µl BCIP/NBT solution; prepared by adding 66 µl of NBT (containing 50 µg/ml nitroblue tetrazolium in 70% N,N-dimethylformamide) and 66 µl of BCIP (50 µg/ml 5-bromo-4-choloro-3-indoyl phosphate in 100% N,N-dimethylformamide) in alkaline phosphate buffer (containing 5.8 g NaCl, 0.1 g $MgCl_2$, 12.1 g Tris). The plates were incubated for 20–25 min at room temperature (25°C) until blue spots developed. The plates were thoroughly washed with running tap water and air-dried for 24 hours.

Blue spots showing fuzzy borders were considered positive. Both positive and negative cells were counted microscopically and the results were interpreted in terms of per cent antibodies secreting cells.

Statistical analysis

Data was analysed by using the complete randomized design and means were compared by Duncan's multiple range test (Steel et al., 1996).

RESULTS

Irrespective of the vaccine used, the number of IgG antibody secreting cells (ASC) in the spleen of orally vaccinated chickens was higher than the number of IgA and IgM ASC. No ASC (positive spots) were seen in control wells for IgA and IgM; although 2–3 false positive spots lacking the characteristic dark centre and granular appearance were observed in some of the control wells for IgG ASC. The number of ASC (IgG, IgA and IgM) per 10^4 cells in spleen was significantly higher ($P < 0.05$) in Group III as compared to Group II and Group I (Table 1).

Maximum protection was seen in Group III (77.8%) followed by Group II (55.6%), and Group I (52.0%). Per cent mortality in experimental and control groups is shown in Figure 1. The results demonstrated a significant difference ($P < 0.05$) in per cent morality between the vaccinated and unvaccinated (control) chickens. However, among vaccinated groups of chickens, significantly lower mortality ($P < 0.05$) was recorded in Group III as compared to Group I and Group II; the difference between Group I and Group II was insignificant ($P > 0.05$). Further, vaccinated chickens were nor-

Table 1. Number of antibody secreting cells (ASC) per 10^4 splenocytes; each value presents the mean ± SD

Groups	ASC/10^4 splenocytes (%)		
	IgA	IgG	IgM
I	870 ± 32^b (8.7)	1 310 ± 57^b (13.1)	450 ± 21^b (4.5)
II	1 250 ± 61^b (12.5)	1 310 ± 77^b (13.1)	690 ± 49^b (6.9)
III	2 360 ± 146^a (23.6)	2 950 ± 130^a (29.5)	1 390 ± 92^a (13.9)

The mean values within a column showing different letters are significant ($P < 0.05$)

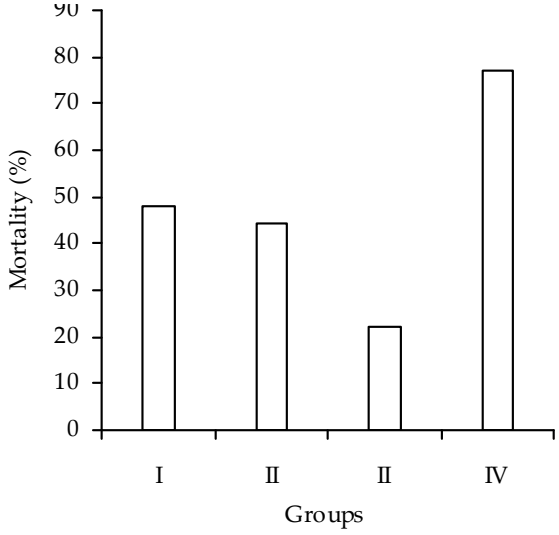

Figure 1. Per cent mortality in vaccinated and control groups after challenge with mixed species of *Eimeria* sp.

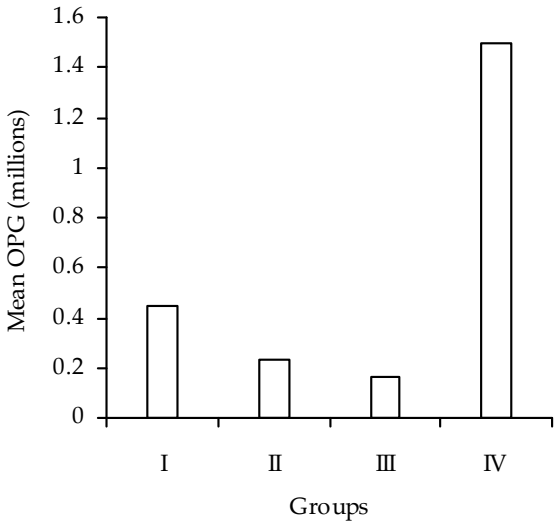

Figure 2. Oocysts per gram (OPG) of droppings in vaccinated and control groups after challenge with mixed species

mal, showing no signs/symptoms of coccidiosis as compared to the controls that were depressed, with ruffled feather. On post mortem examinations, extensive hemorrhagic lesions on the intestine and caeca filled with blood were found in the control chickens. The caeca of the vaccinated chickens contained no blood although very mild lesions on the intestines as compared to the control group were seen.

Mean oocysts per gram (OPG) of droppings were maximum in the control group (D) and minimum in vaccinated groups (I, II and III) as shown Figure 2; the difference was significant ($P < 0.05$). The maximum reduction in the oocyst count was recorded in Group III (88%) followed by Group II (84%) and Group I (70%).

The intestines and caeca of the survived and dead chickens during challenge experiments were subjected to lesion scoring. Minimum lesion scoring was observed in Group III followed by Group II and Group I (Table 2).

DISCUSSION

Immunoglobulins, including IgG, IgA, and IgM, play a vital role in the binding of foreign antigens and the presence of these antibody molecules on a microbial or parasitic surface can cause clumping (agglutination), and among them IgG and IgM activate the complement system (Tizard, 1998; Reinap et al., 1999). It is difficult to understand the complex

Table 2. Lesion scoring on Day 21 post inoculation

Groups	Number of chickens with lesions	Lesion scoring				
Scoring →		0	1	2	3	4
Group I (n = 35)	33[a]	2	5	4	2	22
Group II (n = 33)	30[a]	3	4	3	3	20
Group III (n = 23)	17[b]	6	5	5	1	10
Group IV (n = 49)	46[c]	3	6	3	2	35

0 = no lesion; 1 = scattered lesions; 2 = marked bleeding lesions; 3 = extensively developed lesions with the thickening of intestinal/caecal mucosa; 4 = bloody intestinal/caecal contents

The values within a column showing different letters are significant ($P < 0.05$)

molecular and cellular events involved in intestinal immunity in coccidiosis although it has been reported that chickens infected with *Eimeria* species produce parasitic specific antibodies both in circulation and mucosal secretions (Yun et al., 2000). It has been assumed that antibodies probably develop in parallel with the cellular immunity (Morita et al., 1972; Sanda, 1977; Davis et al., 1985; Long et al., 1986; Bedrink and Kucera, 1988) and participate in protection *per se* and, possibly, as modulators of cellular responses (Mockett and Rose, 1986). In our previous experiments, IgG antibodies were detected by ELISA in chickens vaccinated with *E. tenella* (local isolates) gametocyte vaccine (Hafeez et al., 2006). The present paper reports on the IgA, IgG and IgM antibodies secreting cells in the spleen of chickens vaccinated with *E. tenella* gametocyte vaccine(s) by using ELISPOT assay.

The maximum number of IgG, IgA and IgM ASC was found in Group III followed by Group II and Group I. However, the number of ASC in the present study was comparatively low as reported in mice and rats (Van Lovern et al., 1988; Xu et al., 1991), possibly due to the nature of the antigen used and route of administration. Chickens used in the present study were immunized orally with *E. tenella* gametocyte antigen, and thus the large number of ASC might not be induced.

In our previous studies, secretory IgA antibodies producing cells were also detected in chickens vaccinated with egg adapted gametocyte antigen (Akhtar et al., 2002b). In the present study, such a high level of immunoglobulin secretors in chickens vaccinated with formalin inactivated sonicated gametocyte vaccine (Group III) indicated the synthesis of major immunoglobulins particularly IgA and IgG that may prevent the adherence of the coccidial parasite to the body of the host (Tizard, 1998) and may provide protection against mixed species of the genus *Eimeria* in our present and previous studies (Hafeez et al., 2006). The high number of IgG ASC in comparison with IgM and IgA suggested a role for IgG in local protection against *Eimeria* in chickens (Girard et al., 1997). Further, IgG (IgY) antibodies are known to be concentrated in the egg yolk in the laying hens and are thus considered to be of some relevance in maternal immunity (Rose, 1971, 1972; Wallach et al., 1992; Smith et al., 1994a,b; Wallach, 1997). We have reported that the vaccine containing egg propagated gametocytes of *E. tenella* can induce protective immunity against *E. tenella* infections in offspring chicks (Hafeez et al., 2007). In the present study, a considerable number of IgA ASC was detected in the spleen of immunized chickens, which may produce parasite specific IgA. Based on these results, it can be assumed that the spleen in chickens is one of the major sources of cell producing IgA, IgG and IgM antibodies.

Lesion scores were minimum in Group III followed by Group II and Group I. They were directly proportional to the mortality and OPG of droppings but inversely proportional to the per cent protection. It was also observed that mild to moderate lesions were produced, despite of the immunity conferred that may not lead to fatality to cause the disease or mortality (Conway et al., 1990, 1993, 1999).

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REFERENCES

Akhtar M., Ayaz M.M., Hayat C.S., Ashfaque M., Hussain I. (2002a): Adaptation of *Eimeria tenella* sporozoites (local isolate) in the embryonated hen's eggs. Pakistan Veterinary Journal, 22, 40–41.

Akhtar M., Ayaz M.M., Hayat C.S., Jabbar A., Hafeez M.A., Ashfaque M., Hussain I. (2002b): ELISPOT assay for detection of IgA secreting splenic cells in chicken. Online Journal of Biological Sciences, 2, 554–555.

Ayaz M.M. (2003): Development of egg adapted vaccine (local isolate) against coccidiosis in poultry. [PhD thesis.] University of Agriculture, Faisalabad, Pakistan.

Bedrink P., Kucera J. (1988): Development of immunity against coccidiosis in broiler following administration of oocysts by different routes (in Czech). Biologizace a Chemizace Zivocissne Vyroby-Veterinaria, 24, 507–514.

Bradford M.M. (1976): A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, 72, 248.

Conway D.P., Mckenzie M.E., Dayton A.D. (1990): Relationship of coccidial lesion scores and weight gain in infections of *E. acervulina*, *E. maxima* and *E. tenella* in broilers. Avian Pathology, 19, 489–496.

Conway D.P., Sasai K.S., Gaffar M., Smothers C.D. (1993): Effects of different levels of *Oocyst* inocula of *Eimeria acervulina*, *Eimeria tenella* and *E. maxima* on plasma constituents, packed cell volume, lesion scores and performance in chickens. Avian Diseases, 37, 118–123.

Conway D.P., Dayton A.D., Mckenzie M.E. (1999): Comparative testing of anticoccidials in broiler chickens; the role of coccidial lesion scores. Poultry Science, 78, 529–535.

Davis P.J., Barrat M.E.J., Morgan M., Parry S.H., McDonald L.R., Joyner L.P., Long P.L. (1985): Immune response of chickens to oral immunization by trickle infection with *Eimeria*. Research in Avian Coccidiosis. In: Proceedings Georgia Coccidiosis Conference, 18–20 November, 618–633.

Fu H.M., Lee Y.C. (1976): Immunological studies on chemically attenuated oocysts of chicken caecal coccidisisi. Journal of Chinese Society of Veterinary Science, 2, 51–55.

Girard F., Fort G., Yvore P., Quere P. (1997): Kinetics of specific immunoglobulin A, M and G production in the duodenal and caecal mucosa of chickens infected with *E. acervulina* or *E. tenella*. International Journal of Parasitology, 27, 803–809.

Hafeez M.A., Akhtar M., Hussain I. (2006): Protective effect of egg propagated *Eimeria tenella* (local isolates) gametocytes as vaccine(s) against mixed species of coccidian in chickens. Parasitology Research, 98, 539–544.

Hafeez M.A., Akhtar M., Javed M.T., Haq A.U. (2007): Maternal immunization by egg propagated gametocyte vaccine to control *Eimeria tenella* infections in newly hatched chicks. Parasitology Research, 100, 1139–1142.

Hein H. (1970): Pathogenic effect of *E. necatrix* in young chickens. Experimental Parasitology, 30, 329–335.

Jeurissen S.H., Janes E.M., Vermeulen A.N. (1989): Lymphoid and non-lymphoid cells in the caecal mucosa of naïve and immune chickens related to the development of *E. tenella.* In: Coccidiosis and Intestinal Coccidiomorphs. Vth International Coccidiosis Confference, INRA, Tours, 551–556.

Johnson J., Reid W.M. (1970): Anticoccidial drugs: Lesion scoring techniques in battery and floor pen experiments with chickens. Experimental Parasitology, 28, 30–36.

Lebacq-Verheyden A.M., Vaerman J.P., Heremans J.F. (1972): A possible homologue of mammalian IgA in chicken serum and secretions. Immunology, 22, 165–175.

Lillehoj H.S., Trout J.M. (1996): Avian gut associated lymphoid tissues and intestinal immune responses to *Eimeria* parasites. Clinic Microbial Review, 9, 349–360.

Long P.L., Jahson J., McKenzie M.E., Perry E., Crane M.J., Murany P.K. (1986): Immunization of young broiler chickens with low level of infection of *E. tenella, E. acervalina* or *E. maxima*. Avian Pathology, 15, 271–278.

Mockett A.P.A., Rose M.E. (1986): Immune responses to *Eimeria*: quantification of antibody isotypes to *E. tenella* in chicken serum and bile by mean of the ELISA. Parasite Immunology, 8, 481–489.

Morita C., Tsutsumi Y., Soekawa M. (1972): Detection of humoral antibody for *Eimeria tenella* infection by a dye test. Zentralblatt fur Veterinar Medizin, 19, 782–784.

Reinap R.A., Danforth H.D., Elroy A.P.M., Jones C.B., Caldwell D.J. (1999): Measurement of possible indicators of coccidial immunity in chickens: Responsiveness of mucosal epithelium to *Eimeria* antigens following immunization. In: 20th Annual Meeting, Southern Poultry Science Society, January 18–19, p. 108.

Rose M.E. (1971): Immunity to coccidiosis: protective effect of transferred serum in *Eimeria maxima* infections. Parasitology, 62**,** 11–25.

Rose M.E. (1972): Immunity to coccidiosis: maternal transfer in *Eimeria maxima* infections. Parasitology, 65**,** 273–282.

Ryley J.F., Meade R., Hazelhurt J., Robinson T.E (1976): Methods in coccidiosis research: separation of oocysts from faeces. Parasitology, 73, 311–326.

Sanda A. (1977): Development of immunity to coccidiosis caused by *E. tenella* in day old chicks. Acta Veterinaria, 46, 311–314.

Smith N.C., Wallach M., Miller C.M., Braun R., Eckert J. (1994a): Maternal transmission of immunity to Eimeria maxima: western blot analysis of protective antibodies induced by infection. Infection and Immunity, 62, 4811–4817.

Smith N.C., Wallach M., Miller C.M., Morgenstern R., Braun R., Eckert J. (1994b): Maternal transmission of immunity to *Eimeria maxima*: enzyme-linked immunosorbent assay analysis of protective antibodies induced by infection. Infection and Immunity, 62, 1348–1357.

Speer C.A., Hammond D.M., Mahrt J.L., Roberts W.L. (1973): Structure of the o*ocysts* and sporocyst walls and excystation of sporozoites of *Isospora canis*. Journal of Parasitology, 59, 35–40.

Steel R.G.D., Torrie J.H., Dickey D.A. (1996): Principles and Procedures of Statistics: a Biometrical Approach. 3rd ed. McGraw-Hill, San Francisco.

Tizard I.R. (1998): Veterinary Immunology; an Introduction. 5th ed. Indian Ed. Indra Printers, New Dehli, India. 254–264.

Van Lovern H.A.D., Osterhaus M.E., Nagel J., Schurman H.J., Vos J.G. (1988): Detection of IgA antibodies and quantification of IgA antibody producing cells specific for ovalbumin or *Trichinella spiralis* in the rat. Scandinavian Journal of Immunology, 28, 377–381.

Vervelde L., Vermeulen A.N., Jeurissen S.H. (1992): *In situ* immunocytochemical detection of cells containing antibodies specific for *Eimeria tenella* antigens. Journal of Immunological Methods, 151, 191–199.

Wallach M. (1997): The importance of transmission-blocking immunity in the control of infections by apicomplexan parasites. International Journal of Parasitology, 27, 1159–1167.

Wallach M., Halabi A., Pillemer G., Sar-Shalom O., Mencher D., Gilad M., Bendheim U., Danforth H.D., Augustine P.C. (1992): Maternal immunization with gametocyte antigens as a means of providing protective immunity against *Eimeria maxima* in chickens. Infection and Immunity, 60, 2036–2039.

Xu Y., Macedonia J., Sher A., Pearce E., Cheever A.W. (1991): Dynamic analysis of splenic Th1 and Th2 lymphocyte functions in mice infected with *Schistosoma japonicum*. Infection and Immunity, 59, 2934–2940.

Yun C.H., Lillehoj H.S., Lillehoj E.P. (2000): Intestinal immune responses to coccidiosis. Developmental and Comparative Immunology, 24, 303–324.

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