Applied Biological Research 18 (2): 178-184; (2016)
DOI: 10.5958/0974-4517.2016.00027.6

HAEMATO-BIOCHEMICAL PROFILE AND IMMUNITY TO 12th PASSAGED *Eimeria* spp. OOCYSTS IN FOWL (*Gallus domesticus*)

Birendra Kishor[1*] and M.A. Quadri[2]

[1,2]*Department of Veterinary Parasitology, College of Veterinary Science and Animal Husbandry, Birsa Agricultural University, Ranchi - 834 006, Jharkhand (India)*
[2]*International Institute of Veterinary Education and Research (IIVER), NH-10 Bahu Akbarpur, Rohtak - 124 001, Haryana (India)*
*e-mail: *kishorbiru@gmail.com; afeefquadri@gmail.com*

(Received 23 April, 2015; Accepted 27 June, 2016)

ABSTRACT

The present study was conducted in 24 fowls (*Gallus domesticus*) randomly divided into 3 groups. Group I was kept as negative control. Group II, positive control, was induced with 5000 field strain of sporulated *Eimeria* spp. oocysts and group III induced with 5000 passages strain of *Eimeria* spp. oocysts. On day 21, group II and III were challenged with homologous virulent strain of *Eimeria* spp. Blood and faecal samples were collected at days 26, 31 and 36. One bird from each group was sacrificed on day 28 for lesion scores. All haematological parameter differed significantly, except basophils. Serum transaminases, glucose, body weight and oocyts g^{-1} showed significant change. Heavy lesion score appeared in group II. High stimulation index value was found on day 31 and distinct band of precipitation was observed in group III. The passaged oocysts feeding might be useful in protecting mixed *Eimeria* species infection in fowls.

Key words: Fowl, *Eimeria* spp., Hemato-biochemical, attenuated strain, immunity

INTRODUCTION

Coccidiosis is an economically important disease in poultry worldwide (Singla and Gupta, 2012). It is currently controlled by medication, but increasing emergence of drug-resistant strains of coccidia urges development of alternative control strategies (Pangasa and Singla, 2007). The development of an effective coccidia vaccine should be feasible since natural infection by this parasite is self-limiting, displays minimum antigenic variation and induces long lasting immunity. Attenuated coccidia strains have been successfully produced through rigorous selection of precociousness and vaccination programmes using them are being actively pursued. Numerous recombinant coccidial antigens have been characterized and experimental immunization studies are promising (Lillehoj, 1991). However, practical application of recombinant vaccines to the poultry industry is still remote and a better understanding of mucosal immunity may be crucial before their application becomes meaningful (Lillehoj and Trout, 1993).

Live vaccines consisting of attenuated parasites have provided a measure of control for avian coccidiosis (Vermeulen *et al*., 2003). The present study is an attempt to assess host immunity and develop attenuated vaccine to coccidia by passage oocysts feeding, which could be useful in protecting mixed *Eimeria* species infection in fowls under natural field conditions.

MATERIALS AND METHODS

Preparation of selected strain of Eimeria spp. for passage

The birds having *Eimeria* spp. infection and history of a normal sign and symptoms were used for the collection of *Eimeria* spp. for passage. Oocysts obtained from the field faecal samples were kept in 2.5% $KMnO_4$ solution at 29 ± 1^0C for sporulation. The sporulated oocysts were separated by oocyst floatation method (Devi *et al.*, 2010). Sporulated oocysts were inoculated directly into the crop of chicken with a narrow rubber tube attached syringe. When oocysts began to appear in their faeces, the sample was collected from 5^{th} to 10^{th} day after inoculation from live birds, separately. The faecal materials were treated as above for sporulation and oocysts separation. Likewise, 12 passage oocysts were obtained after infecting fresh groups of birds each time. After 12^{th} passage plated oocysts were stored in phosphate buffer saline at 4^0C until experimentation.

One day old chicks were brought from a hatchery. On day 21^{st} the birds were randomly divided into 3 groups each containing 8 birds. Feed and water were allowed *ad-libitum* to all birds throughout the period of experiment. Group I was reared as non-infected and non-medicated control group. Group II was kept as infected with 5,000 sporulated oocysts, non-medicated control group. The birds of group III were inoculated with 5,000 oocysts selected/passages strain of *Eimeria* spp. After 21^{st} days after inoculation all birds, except group I, were challenge with virulent *Eimeria* spp. sporulated ($2X10^4$) oocysts.

The haematological observations *viz*., haemoglobin, packed cell volume, total leukocyte count and differential leukocyte count were made as per Schalm *et al*. (1975). Serum glucose, total protein, ALT and AST were estimated by Auto analyzer (15 Biosystem) using COGENT diagnostic kit (Span diagnostics Ltd.). The oocysts were estimated as per Stoll (1930). The body weight and humoral immunity response was assessed by agar gel precipitation test and cell-mediated immune response by lymphocyte stimulation test (LST) on day 26, 31 and 36 day post-infection. One bird from each group was sacrificed on 28^{th} day to know the lesion score in intestine including caeca of infected and treated groups.

Immunological studies

For preparation of *Eimeria* spp. antigens, oocysts were disrupted by the sonicator. The protein concentration of antigen was determined (Lowry *et al*., 1951) and final concentration was adjusted to 250 µg protein mL^{-1} by phosphate buffer saline.

Humoral immune response: The double diffusion method was used (Ouchterlony, 1953). Agar was made up as 1.5% solution in 8% NaCl buffered with veronal at pH 7.4. A standard volume was poured on to a lantern slide, retained within a nickel-plated ring. The high salt concentration was used to satisfy the requirement for fowl antibody for complete precipitation (Goodman *et al*., 1951). The plates were allowed up to I4 days for the full development of the reaction.

Cell-mediated immune response: For separation of peripheral blood mononuclear cells, blood (5 mL) from bird was collected in heparinised and sterilized test tube. It was then diluted in 10 mL of RPMI-1640 media (Rosewell Park Memorial Institute-1640) containing 5% foetal calf serum. Diluted blood (8 mL) was carefully overlaid over 4 mL lymphocyte separation fluid (HiSep LSM, HiMedia Laboratories, Mumbai) in a centrifuge tubes and centrifuged at 400 x g for 30 min. White layer containing mononuclear cells at interface of plasma and lymphocyte separation fluid was collected with the help of pasture pipettes. The collected cells were washed twice with RPMI-1640 culture media at 400 x g for 10 min. The resulting pellets were suspended in double volume of RPMI-640 media. Cell viability was ascertained by trypan blue dye exclusion method. Finally, the cell concentrations were adjusted to $2 X 10^6$ cells mL^{-1} medium.

3-[4, 5 Dimethylthiazole-2-4] 2, 5-diphenyl tetrazolium bromide (MTT) assay

Lymphocyte proliferative assay was performed using 3-[4, 5 dimethylthiazole-2-4] 2, 5-diphenyl tetrazolium bromide (MTT, Sigma-Aldrich, UK) dye using by Mosmann (1983) method with some modifications. Briefly, 180 µL cell suspension from each bird was dispensed in well tissue culture plates. Then 20 µL medium containing mitogen (Con A @ 40 µg mL^{-1}) was added in wells. Correspondingly, 20 µL medium containing antigen [oocysts of coccidia @ 40 µg mL^{-1} RPMI-1640 medium] was added in wells of tissue culture plate. In the remaining wells, 20 µL serum-free RPMI-1640 medium was added which acted as control wells. The plates were incubated at 37^0C in CO_2 incubator for 68 hr. After incubation, 20 µL MTT dye (5 mg mL^{-1} medium) was added in each well. The tissue culture plate was re-incubated at 37°C for 4 hr in CO_2 incubator. After incubation, 100 µL supernatant was removed carefully from each well without disturbing Formosan precipitates. Then 100 µL MTT stop solution (10 mL DMSO + 300 µL Triton X-100) was added to each well and mixed to dissolve Formosan precipitates completely. Optical density (OD) was taken at 540 nm in ELISA reader (BioRaid, USA). The results of lymph proliferative assay were expressed as stimulation index (SI) using the formula:

$$\text{S.I.} = \frac{\text{Mean OD of stimulated cell culture}}{\text{Mean O.D. of unstimulated cell culture}}$$

Lesion scoring was done by scarifying on 28^{th} day of experiment in both groups as per Johnson and Reid (1970). The data was statistical analyzed using Duncan's multiple range test (Snedecor and Cochran, 2004).

RESULTS AND DISCUSSION

Significant ($P<0.05$) decrease in haemoglobin and PCV within days and groups was the marked feature of infection (group II) {Table 1}. In group III, the Hb and PCV level were comparable to control at day 36 which indicted that the challenge is more effective method to avoid ill effect. Hirani *et al.* (2007) and Patra *et al.* (2010) reported haemoglobinaemia and reduced packed cell volume during coccidia infection. In our study a significant increase was recorded in TLC of group II which was high at day 36. TLC of group III was comparable with healthy group. Increase in lymphocytes was significant and found time-dependent in group II. Eosinophils also increased significantly in group II, whereas heterophils and monocytes showed significant decrease in time-dependent manner in group II. No change in basophil count was recorded which indicated no allergic response / symptoms. Fowls of group III showed all haematological values within normal range at day 36. Hirani *et al.* (2007) also observed similar findings.

Group II infected with *Eimeria* spp. had significantly ($P<0.05$) high serum glucose, aspartate transaminase, alanine transaminase; whereas total serum protein was significantly reduced. The increase/decrease was time dependent. The challenge of infection with virulent strain (group III) maintained all the values within normal limits. These values were in congruence with the literature (Hirani *et al.*, 2007*;* Pangasa *et al.*, 2007; Patra *et al.*, 2010; Mondal *et al.*, 2011). The alterations in biochemical profile might be due to dysfunction of liver caused by the invasion of parasite (*Eimeria* spp.) during infection and subsequent recovery of organ after inoculation of passage oocysts indicated an immunogenic effect of parasite. Being primarily cellular enzymes of hepatocytes, ALT and AST increase is an important marker of liver damage. They were also present in other tissues including intestine, hence the increase in ALT was rendered to hepatic disturbance mediated due to coccidial enteritis. Moreover, it could also be due to degenerative changes in intestinal mucosa.

The body weights significantly increased ($P<0.05$) in all groups though a high growth rate was observed in group I (healthy control) followed by group III. The lowest body weight was recorded in group II (infected un-treated). Lee *et al.* (2009) reported higher body weight gain in

Table 1: Hemato-biochemical parameters and immunity in fowls (*Gallus domesticus*) immunized and challenged with *Eimeria* spp. oocysts on various days of observations

	Parameters	Days	Groups		
			I	II	III
Haemogram	Hb (gm %)	26	10.66 ± 0.12^{C}	5.31 ± 0.41^{bA}	9.09 ± 0.21^{aB}
		31	10.70 ± 0.13^{C}	4.35 ± 0.29^{aA}	9.91 ± 0.15^{bB}
		36	11.08 ± 0.16^{B}	3.66 ± 0.21^{aA}	10.76 ± 0.16^{cB}
	PCV (%)	26	33.51 ± 0.30^{C}	13.97 ± 0.36^{cA}	28.80 ± 0.61^{aB}
		31	33.65 ± 0.14^{C}	10.14 ± 0.35^{bA}	31.60 ± 0.69^{bB}
		36	33.80 ± 0.12^{C}	8.80 ± 0.17^{aA}	32.50 ± 0.35^{bB}
	TLC (10^3 μL^{-1})	26	10.93 ± 0.19^{A}	13.99 ± 0.10^{aB}	11.35 ± 0.28^{A}
		31	10.95 ± 0.19^{A}	14.23 ± 0.14^{aB}	11.13 ± 0.16^{A}
		36	10.98 ± 0.21^{A}	17.03 ± 0.34^{bB}	11.06 ± 0.26^{A}
	Lymphocytes (%)	26	56.25 ± 0.67^{A}	66.75 ± 0.80^{aC}	60.75 ± 0.53^{bB}
		31	56.38 ± 0.50^{A}	74.38 ± 0.49^{bC}	58.49 ± 0.71^{bB}
		36	56.50 ± 0.50^{A}	78.13 ± 0.46^{cB}	56.50 ± 0.85^{aA}
	Heterophils	26	30.50 ± 0.68^{C}	19.25 ± 0.59^{cA}	26.25 ± 0.70^{aB}
		31	29.88 ± 1.47^{B}	12.00 ± 0.49^{bA}	28.50 ± 0.73^{abB}
		36	30.88 ± 0.79^{B}	9.75 ± 0.65^{aA}	29.75 ± 1.09^{bB}
	Eosinophils (%)	26	1.75 ± 0.16^{A}	2.75 ± 0.19^{B}	1.88 ± 0.14^{A}
		31	1.50 ± 0.19^{A}	2.75 ± 0.17^{B}	1.75 ± 0.17^{A}
		36	1.38 ± 0.18^{A}	3.00 ± 0.31^{B}	1.50 ± 0.22^{A}
	Monocyte (%)	26	9.50 ± 0.19	9.63 ± 0.18^{b}	9.50 ± 0.19
		31	9.63 ± 0.18	9.00 ± 0.29^{ab}	9.63 ± 0.20
		36	9.88 ± 0.30^{B}	8.38 ± 0.30^{aA}	9.50 ± 0.22^{B}
	Basophils (%)	26	1.63 ± 0.18	1.75 ± 0.16	1.63 ± 0.18
		31	1.63 ± 0.18	1.88 ± 0.24	1.63 ± 0.20
		36	1.50 ± 0.19	2.00 ± 0.22	1.75 ± 0.19
Biochemical	Serum ALT (I.U)	26	7.05 ± 0.18^{A}	8.41 ± 0.17^{aB}	7.26 ± 0.14^{A}
		31	7.09 ± 0.16^{A}	8.88 ± 0.13^{bB}	7.18 ± 0.14^{A}
		36	7.10 ± 0.08^{A}	9.10 ± 0.12^{bB}	7.13 ± 0.15^{A}
	Serum AST (I.U)	26	65.10 ± 0.35^{A}	79.80 ± 0.32^{aC}	69.60 ± 0.41^{bB}
		31	65.50 ± 0.21^{A}	88.40 ± 0.45^{bC}	68.31 ± 0.50^{aB}
		36	65.69 ± 0.27^{A}	97.38 ± 0.69^{CC}	67.14 ± 0.37^{aB}
	Total serum protein ($g\ dL^{-1}$)	26	4.95 ± 0.19^{C}	2.90 ± 0.20^{bA}	4.25 ± 0.14^{aB}
		31	4.96 ± 0.17^{B}	2.51 ± 0.13^{abA}	4.79 ± 0.14^{bB}
		36	4.99 ± 0.14^{B}	2.15 ± 0.17^{aA}	4.89 ± 0.15^{bB}
	Serum glucose ($mg\ dL^{-1}$)	26	179.85 ± 1.74^{A}	226.80 ± 2.20^{aB}	185.13 ± 1.38^{bA}
		31	180.10 ± 1.69^{A}	238.53 ± 2.07^{bB}	182.36 ± 1.22^{abA}
		36	180.64 ± 1.81^{A}	247.14 ± 2.97^{cB}	181.15 ± 1.13^{aA}
	Body weight	26	1.92 ± 0.06^{aC}	1.45 ± 0.006^{aA}	1.79 ± 0.048^{aB}
		31	2.45 ± 0.05^{bC}	1.69 ± 0.016^{bA}	2.28 ± 0.037^{bB}
		36	2.85 ± 0.05^{cC}	1.88 ± 0.035^{cA}	2.61 ± 0.049^{cB}
Parasitological	Oocysts (g^{-1})	26	-	24937.5 ± 276.42^{aA}	26612.5 ± 271.53^{cB}
		31	-	37012.5 ± 382.40^{bB}	14562.5 ± 194.51^{bA}
		36	-	42337.5 ± 475.82^{cB}	8275.0 ± 252.66^{aA}
Immunological	Lymphocyte antigen stimulation index	26	1.60 ± 0.055^{aA}	2.065 ± 0.095^{bC}	1.806 ± 0.043^{bB}
		31	2.00 ± 0.097^{bA}	2.303 ± 0.042^{cB}	2.102 ± 0.064^{cAB}
		36	1.70 ± 0.098^{aAB}	1.805 ± 0.041^{aB}	1.606 ± 0.035^{aA}
	lesion scores	31	0	++++	0

Values with same superscripts in a column (lower case) and a row (upper case) do not differ significantly (P<0.05)

immunized group (P<0.05) as compare to the untreated control. Saad *et al.* (2009) observed that vaccinated and challenged by *Eimeria* species had gain body weight as compared to no-vaccinated birds. The body weight alteration might be due to loss of blood through bloody diarrhoea, decreased

feed conversion ratio and weakness in infected group. The present study revealed that the birds of 12th passage oocysts and challenged with field strain had more body weight gain than the birds infected with field strain of infected bird (group III). Dong *et al.* (2006) reported after 16th successive passages oocysts of *E. necatrix* may be use for the development of new coccidiosis vaccine. The present finding also suggested that for the development of complete attenuated line of *Eimeria* spp. in Ranchi zone more passages are required. Immunity to oocysts of *Eimeria* spp. has been reported by Jorgensen (2006) and Mc-Donald and Shirley (2009).

In poultry having infection of 12th passage oocysts group III and group II (infected control) and challenged with virulent strain, the effect of passage was evaluated by observing pre- and post-treatment OPG count. The findings indicated that the OPG of passage birds significantly increased on 26th day in group III; thereafter it decreased significantly, whereas OPG of group II showed significant increase at 36th day of experiment as compared to 26th and 31th days. The OPG of 12th passage group increased at 26th day and thereafter declined. Our findings are supported by Rose (1975) who reported immunized had higher oocysts production as compression to non-immunized birds. Lee *et al.* (2009) reported reduce oocysts output in immunized group as compare to untreated control group during observation on oral infection with *E. tenella* and *E. maxima*.

The stimulation index was maximum at 31st day in group I and II followed by 36th and 26th days, whereas in group III the stimulation index was highest in 31st day followed by 26th day and 36th day. A significant difference ($P<0.05$) was observed between group and days. Similar to our observations, Miyamoto *et al.* (2002) reported decrease in splenic mitogenic response of chickens infected with *Eimeria* transiently at day 7 but it increased significantly at day 10 after primary infections.

The lesion score group II was heavy whereas no lesion was found in group I and III (Fig. 1 and 2). Crouch *et al.* (2003) reported that vaccinated birds, challenged independently on 28th days later with virulent strain of *Eimeria* spp., had associated reduction in lesion formation. Weber *et al.* (2004) reported that chicks immunized by *in ovo* injection of 5 *Eimeria* species oocysts had significantly reduced lesion scores as compared to their nonimmunized counterparts.

Precipitin bands developed as a result of infection. There was no precipitation band in agar gel precipitation test (AGPT) in healthy group, whereas deflection of band was observed in infected group II. In group III a distinct band of precipitation observed. Similar results were obtained by Pierce *et al*. (1962) who demonstrated precipitating antibodies in some but not all of these immune fowls by agar-gel diffusion technique.

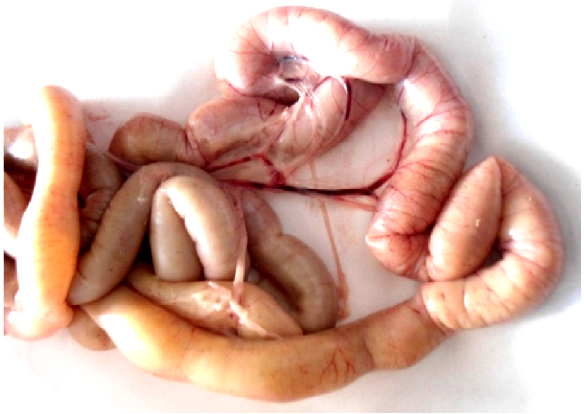

Fig. 1: Showing normal intestine of fowl (lesion score: 0)

Fig. 2: Showing severe hemorrhagic enteritis (lesion score: +4)

Conclusion: The passage oocysts was found useful in lowering the virulence of *Eimeria* species protozoa and could be used as an immunogen for protecting from acute *Eimeria* spp. infection in domestic fowls under natural field conditions.

Acknowledgment: Authors are thankful to the Dean, College of Veterinary Science and Animal Husbandry, Ranchi, for providing the necessary facilities to conduct the preset study.

REFERENCES

Crouch, C.F., Andrews, S.J., Ward, R.G. and Francis, M.J. 2003. Protective efficacy of a live attenuated anti-coccidial vaccine administered to 1-day-old chickens. *Avian Pathol*ogy, **32**: 297-304.

Devi, I.K., Devi, Y.G., Chanu, K.V., Ali, M.A., Tiwari, J.G., Prampha, M., Malsawmkima, D. and Patra, G. 2010. Transmission electron microscopy (TEM) study on the oocyst of *Eimeria tenella*. *International Journal of Poultry Science*, **11**: 1050-1055.

Dong, H., Suo, X., Wang, M. and Teng, K. 2006. Characteristics of a line of *Eimeria necatrix* after 16 successive passages of oocysts collected after peak oocysts production. *Journal of Parasitol*ogy, **92**: 1229-1234.

Goodman, M., Wolfe, H.R. and Norton, S. I951. Precipitin production in chickens VI. Effect of varying concentrations of sodium chloride on precipitate formation. *Journal of Immunology*, **66**: 225-236.

Hirani, N.D., Hasnani, J.J., Dhami, A.J. and Khanna, K. 2007. Haemato-biochemical profile of broilers affected with coccidiosis. *Journal of Veterinary Parasitology*, **21**: 25-28.

Johnson, J. and Reid, W.M. 1970. Anticoccidial drugs lesion scoring techniques in battery and floor pen experiments with chickens. *Experimental Parasitology*, **28**: 30-36.

Jorgensen, W.K., Anderson, G.R., Jeston, P.J., Blight, G.W. and Molloy, J.B. 2006. Selection and characterisation of two attenuated vaccine lines of *Eimeria tenella* in Australia. *Australian Veterinary Journal*, **84(**3): 89-94.

Lee, J.T., Broussard, C., Fitz-Coy, S., Burke, P., Eckert, N.H., Stevens, S.M., Anderson, P.N., Anderson, S.M. and Caldwell, D.J. 2009. Evaluation of live oocysts vaccination or salinomycin for control of field-strain *Eimeria* challenge in broilers on two different feeding programs. *Journal of Applied Poultry Research*, **18**: 458-464.

Lillehoj, H.S. 1991. Cell-mediated immunity in parasitic and bacterial diseases. pp. 155-181. **In**: *Avian Cellular Immunology* (ed. J.M. Sharma). CRC Press, Boca Raton, Florida, USA.

Lillehoj, H.S and Trout, J.M. 1993. Coccidia: A review of recent advances on immunity and vaccine development. *Avian Pathology*, **22:** 3-31.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.I. 1951. Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry*, **193**: 265-275.

Mc-Donald, V. and Shirley, M.W. 2009. Past and future: vaccination against Eimeria. *Parasitology*, **136**: 1477-1489.

Miyamoto, T., Min, W. and Lillehoj, H.S. 2002. Lymphocyte proliferation response during *Eimeria tenella* infection assessed by a new, reliable, nonradioactive colorimetric assay. *Avian diseases*, **46**: 10-16.

Mondal, D.K., Chattopadhyay. S., Batabyal. S., Bera. A.K. and Bhattacharya D. 2011. Plasma biochemical indices at various stages of infection with a field isolate of *Eimeria tenella* in broiler chicken. *Veterinary World*, **4**: 404-409.

Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxic assay. *Journal of Immunological Methods*, **65**: 55-63.

Ouchterlony, O. 1953. Antigen-antibody in gels. IV: Types of reactions in co-ordinated systems of diffusion. *Acta Pathologica Microbiologica Scandinavica*, **32:** 231-241.

Pangasa, A. and Singla, L.D. 2007. Effect of coccidiostats and immunomodulators on haematology of *Eimeria tenella* infected broilers. *Indian Veterinary Journal*, **84:** 1131-1134.

Pangasa, A., Singla, L.D. and Ashuma 2007. Biochemical alterations in chicken during *Eimeria tenella* infection medicated with coccidiostats and immunomodulator. *The Indian Journal for Field Veterinarians*, **3**(2): 06-10.

Patra, G., Ali, M.A., Chanu, K.V., Jonathan, L., Joy, L.K., Prava, M., Ravindran, R., Das, G. and Devi, L.I. 2010. PCR based diagnosis of *Eimeria tenella* infection in broiler chicken. *International Journal of Poultry Science*, **9**: 813-818.

Pierce, A.E., Long, P.L. and Smith, C.H. I962. Immunity to *Eimeria tenella* in young fowls (*Gallus domesticus*). *Immunology*, **5**: 129-152.

Rose, M.E. 1975. Infections with *Eimeria maxima* and *Eimeria acervulina* in fowl. *Parasitology*, **70**: 263-271.

Saad, M.B., Bona, T.D.M.M., Serafini, C.A.V., Romani, F., Alvarez, J.M., Camargo, M.L. and Santin, E. 2009. Evaluating different vaccine routes against Coccidiosis. *International Journal of Poultry Science*, **8**: 980-984.

Schalm, O.W., Jain, N.C. and Carrol, E.J. 1975. *Veterinary Haematology* (3rd edn.). Lea and Febiger Philadelphia, USA.

Singla, L.D. and Gupta. S.K. 2012. Advances in diagnosis of coccidiosis in poultry. pp 615-628. **In**: *Veterinary Diagnostics: Current Trends* (eds. R.P. Gupta, S.R. Garg, V. Nehra and D. Lather), Satish Serial Publishing House, Delhi, India.

Snedecor, G.W. and Cochran, W.G. 2004. *Statistical Method* (8th edn.), Oxford and IBH Publishing Co., New Delhi, India.

Stoll, N.R. 1930. Investigation on the control of hookworm disease. An effective method of counting hook worm eggs in the faeces. *American Journal of Hygiene*, **3**: 59-60.

Vermeulen, A.N. 1998. Progress in recombinant vaccine development against coccidiosis. A review and prospects into next century. *International Journal of Parasitology*, **28:** 1121-1130.

Weber, F.H., Genteman, K.C., Le, M.M.A. Sr, L.D.O and Evans, N.A. 2004. Immunization of broiler chicks by *in ovo* injection of infective stages of *Eimeria*. *Poultry Science*, **83**: 392-399.