ISSN 1682-8356
ansinet.org/ijps

INTERNATIONAL JOURNAL OF

POULTRY SCIENCE

ANSInet
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan
Mob: +92 300 3008585, Fax: +92 41 8815544
E-mail: editorijps@gmail.com

International Journal of Poultry Science 9 (8): 813-818, 2010
ISSN 1682-8356

PCR Based Diagnosis of *Eimeria tenella* Infection in Broiler Chicken

Gautam Patra*, M. Ayub Ali[1], Kh. Victoria Chanu[1], L. Jonathan[2],
L.K. Joy[2], M. Prava[1], R. Ravindran[3], G. Das[4] and L. Inaotombi Devi[5]
*Department of Veterinary Parasitology, [1]Department of Veterinary Physiology and Biochemistry,
[2]SRF-RCRL, [3]Department of Veterinary Pathology, [4]Department of Veterinary Clinical Medicine,
College of Veterinary Sciences and Animal Husbandry,
Central Agricultural University, Selesih, Aizawl, Mizoram, India
[5]Department of MLT, RIPANS, Aizawl, Mizoram, India

Abstract: The present investigation was undertaken to evaluate histo-pathological, haemotological and biochemical changes in broiler chicken naturally infected with *Eimeria tenella*. A Polymerase Chain Reaction (PCR) based assay was also done for the detection, identification and differentiation of pathogenic species of *Eimeria* in poultry. Post mortem examination revealed petechial haemorrhages, oedema, necrosis and sloughing of caecal epithelium. Histopathological evidence showed leakage of blood, oedema and necrosis. Haemotological studies revealed that coccidial infection caused significant decrease in the value of haemoglobin and Packed Cell Volume (PCV). The value of Mean Corpuscular Volume (MCV) and Mean Corpuscular Haemoglobin Concentration (MCHC) revealed that caecal coccidiosis resulted in macrocytic hypochromic anaemia. Biochemical serum analysis of coccidial infected chicken showed a significant increase in level of glucose, Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST) while there was a significant decrease in the level of alkaline phosphatase (SAP) and cholesterol. In PCR using specific primers for *E. tenella* only the ITS 1 regions of ribosomal DNA (rDNA) could be amplified.

Key words: Histopathology, haematology, serum biochemistry, molecular diagnosis, chicken coccidiosis

INTRODUCTION

Coccidiosis is caused by species of intracellular protozoan parasites belonging to the genus *Eimeria* (Phylum Apicomplexa). It is one of the most economically important diseases in poultry in modern poultry production. Infections are controlled by prophylactic medication in feed or by vaccination with live parasites. In domestic chicken seven species of *Eimeria* with different degrees of pathogenicity are recognized viz. *E. acervulina*, *E. brunetti*, *E.maxima*, *E. mitis*, *E. necatrix*, *E. praecox and E. tenella*. During the course of natural infection, several species of *Eimeria* often occur concomitantly (Calnek *et al*., 1997; Shirley, 1999). *Eimeria tenella* is one of the most ubiquitous (Quarzane *et al*., 1998) and most pathogenic (Arakawa and Xie, 1993; Yadav and Gupta, 2001). The lesions caused by this parasite disturb nutrient absorption, triggering several changes in carbohydrates, lipid, protein and macro and trace mineral metabolism (Witlock and Ruff, 1997). Diagnosis of *Eimeria* infections and differentiation between species is usually attempted by consideration of clinical signs in the host and biological features of the parasites. The latter include prepatent period, site of development within the intestine and the morphological appearance of oocysts in the faeces and endogenous stages in the intestinal or caecal mucosa (Joyner and Long, 1974). The presence of parasites may be confirmed by post-mortem examination of the host or by fecal examination.

Recently, Polymerase Chain Reaction (PCR) based amplification of DNA have been used for the diagnosis of coccidial parasites of man and animals. A number of approaches have proved to be both specific and highly sensitive for analyses either of parasites grown in vitro or present in tissue samples and clinical materials, for e.g., *Toxoplasma* (Burg *et al*., 1989; Guay *et al*., 1993) *Neospora* (Holmdahl and Mattsson, 1996; Muller *et al*., 1996) and *Eimeria* (Beate *et al*., 1998; Kawahara *et al*., 2008; Su *et al*., 2003; Lew *et al*., 2003).

An attractive genomic DNA target for PCR analysis is the Internal Transcribed Spacer 1 (ITS 1) gene of ribosomal DNA (rDNA) (Cai *et al*., 1992; Holmdahl and Mattsson, 1996; Jeffries *et al*., 1996; Payne and Ellis, 1996). This spacer separates the 3′ end of the 16S-like ribosomal RNA gene from the 5′ end of the 5.8S rRNA gene within individual rDNA transcription units. Due to its heterogeneity in both sequence length and base composition, the ITS 1 lends itself perfectly for the design of specific primers and as part of the rDNA transcription unit, it is also a member of a multiple copy gene family and thus provides large numbers of potential PCR targets.

Corresponding Author: Gautam Patra, Department of Veterinary Parasitology, College of Veterinary Sciences and Animal Husbandry, Central Agricultural University, Selesih, Aizawl, Mizoram, India

In the present study, an attempt has been made to study the histopathological, biochemical and haematological changes in the birds naturally infected with *E. tenella*. The infection was confirmed by using molecular technique like PCR. The fidelity of the primers pairs was evaluated in tests using DNA samples prepared from purified oocysts of field samples and then tested with each of the four species-specific primers of *E. acervulina, E. brunetti, E. necatrix* and *E. tenella*.

MATERIALS AND METHODS

An outbreak of coccidiosis occurred recently in the broiler chickens aged 3-4 weeks in the instructional poultry farm of College of Veterinary Sciences and Animal Husbandry, Central Agricultural University, Selesih, Aizawl, Mizoram, India with high mortality. On post mortem the gross lesions were recorded. Fresh faecal samples were collected and the oocysts were allowed to sporulate according to the procedures described by Shirley (1995). Blood samples for haematological and biochemical analyses were also collected. One ml of blood was collected in a clean dry test tube containing sodium flouride and another 1.5 ml was collected in plain centrifuge tube and then straw coloured serum was collected after centrifugation at 3000 rpm for 15 min and stored at -20°C for biochemical analysis.

For histopathological examination, tissues from the lesions were collected and fixed in 10% formalin solution. The tissues were then dehydrated in absolute alcohol, cleared in xylene, embedded in paraffin for preparation of fine blocks in paraffin wax; sections of 5µ thickness were cut and stained with routine haematoxylene and eosin staining technique (Lillie, 1965).

DNA extraction: The purified oocysts, stored in 2.5% potassium dichromate solution, were washed 4 times by centrifugation (1.4 x 10^3 rpm for 5 min, each wash) in autoclaved, high-salinity, phosphate buffered saline (300 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.7 mM NaH_2PO_4) (Reece *et al*., 1997). The pellet was resuspended in 200 µl of 5.75% sodium hypochlorite and incubated on ice for 30 min. The oocyst suspension was then diluted with 1 ml of sterile double distilled water and pelleted by centrifugation. The pellet was again washed 3 times with PBS. The pellet was resuspended in 1 ml deionized water and sonicated at high speed sonication using ultrasonicator (OMNI Ruptor400, Made in USA) to disrupt the oocysts. The DNA was extracted from sonicated oocysts and analysed by agarose gel electrophoresis as per standard procedures (Sambrook *et al*., 1989).

Amplification of the ITS1: The genus-specific forward and reverse primer coded `BSEF' and `BSER' of sequence 5^{l}-CTGTGAATCCATCGGA-3^{l} and 5^{l}-ATCGCATTTCGCTGCGTCCT-3^{l} (Bangalore Genei,) respectively was used for the amplification of ITS1 gene as per the methods of Devereux *et al*. (1984).

The PCR product was analysed by gel electrophoresis. The species specific primers for *E. acervulina, E. brunetti, E. necatrix* and *E. tenella* (Bangalore Genei, India) are shown in Table 1.

PCR: The PCR was done in Mastercycler Gradient machine (Eppendrof, Germany), with 50 µl of reaction mixtures for each sample that contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 200 µM of each dNTPs, 100 pmol (20 pmol each of genus specific and species specific, *E. acervulina, E. brunetti, E. necatrix* and *E. tenella*) oligonucleotide primer and target DNA. Another tube of PCR reaction mixture was prepared by using only species specific primer pair, ETF and ETR of *E. tenella*. The optimum temperature for amplification of the DNA was determined by employing different temperature from 57-62°C and subsequently suitable temperature was used for PCR amplification. The amplification procedure was formulated as 94°C for 4.5 min for initial denaturation before adding 1U of Taq DNA polymerase (Bangalore Genei). It is followed by 30 cycles each of denaturation at 94°C for 50 sec, annealing at 62°C for 50 sec and extension step at 72°C for 1 min and the final extension was performed at 72°C for 2 min. Ten-microlitre of the reaction mixtures was electrophoresed in 1.5% agarose gel having ethidium bromide and visualized in a gel documentation system (BioRad, USA).

Table 1: Species specific primer pairs

Primer name	Sequence (5^{l} - 3^{l})	Primer length (bp)	Species
EAF	GGCTTGGATGATGTTTGCTG	20	*Eimeria acervulina*
EAR	CGAACGCAATAACACACGCT	20	
EBF	GATCAGTTTGAGCAAACCTTCG	22	*Eimeria brunette*
EBR	TGGTCTTCCGTACGTCGGAT	20	
ENF	TACATCCCAATCTTTGAATCG	21	*Eimeria necatrix*
ENR	GGCATACTAGCTTCGAGCAAC	21	
ETF	AATTTATCCATCGCAACCCT	20	*Eimeria tenella*
ETR	CGAGCGCTCTGCATACGACA	20	

Table 2: Blood cellular parameters in the normal and infected broiler chicken with *E. tenella*

Parameters	Normal value	Test sample
WBC (10^3/µl)	12-13	58.86
RBC (10^6/µl)	2.5-3.5	0.5
Packed Cell Volume (PCV, %)	22-35	5.9
Haemoglobin concentration ((Hb, g/dl)	7-13	2.5
Mean Corpuscular Volume (MCV, fl)	90-140	7.1
Mean Corpuscular Haemoglobin (MCH, pg)	33-47	50.0
Mean Corpuscular Haemoglobin Concentration (MCHC, g/dl)	26-35	42.3
Lymphocyte (%)	55-60	87.0
Monocyte (%)	10.0	8.1
Granulocyte (%)	30-45	4.9

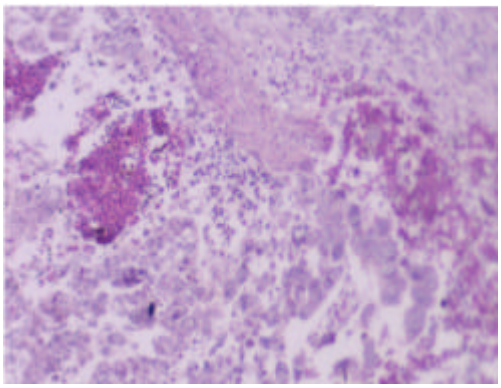

Fig. 1: Section of caeca showing extensive tissue infiltration, haemorrhages and developmental stages of schizonts and oocysts (10x)

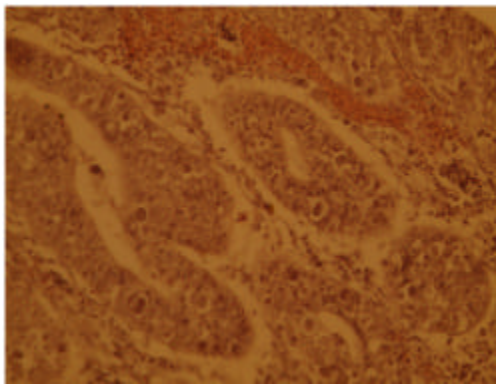

Fig. 2: Section of caeca showing extensive tissue infiltration, haemorrhages and developmental stages of schizonts and oocysts (40x)

RESULTS

All the birds with coccidiosis showed depression, weakness, bloody diarrhoea, anorexia and ruffled feathers. Postmortem examination revealed the distended caeca filled with bloody faeces and mucoid debris with haemorrhages on the mucosa. The bloody mass in the caeca, a characteristic feature of caecal coccidiosis was found in some chicks while others showed mottled reddish or milky white colored contents in the caeca due to formation of oocysts.

Histopathological lesions observed were loss of epithelial tissue, congestion of blood vessels, which indicated disruption followed by leakage of blood. There were also severe muscular oedema, necrosis of mucosa, submucosa of caecum, presence of clusters of oocysts and schizonts and lymphoid cells hyperplasia. Mononuclear cell infiltration was noted in the mucosal layer.

Haematological analysis: Blood cellular analysis of naturally infected broiler chickens with *E. tenella* revealed a significant reduction in Total Erythrocyte Counts (TEC), Haemoglobin (Hb) level and Packed Cell Volume (PCV) with a slightly increase in Mean Corpuscular Haemoglobin (MCH). The differential leukocyte count showed a significant change in lymphocyte and granulocyte count (Table 2).

Biochemical findings: Liver function test of the infected birds showed a significant increase in the serum Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST) and glucose with decrease in albumin, cholesterol and Alkaline Phosphatase (ALP, Table 3). Kidney function test of the infected birds revealed a significantly higher level of uric acid, inorganic phosphorus (Pi), magnesium and chloride (Table 4).

Gene amplification by PCR: The specific annealing temperature of both the genus and species specific primer pairs was found to be 62°C. The amplification of the ITS1 region using the genus and species specific primer pairs at optimum temperature yielded a product of approximately 520 bp and 270 bp respectively as shown in Fig. 3. The primer pair for genus specific and *E. tenella* exclusively amplified their target DNA and there was no cross-species amplification by heterologus primer pairs as shown in Fig. 3 and 4.

DISCUSSION

Coccidiosis in poultry is characterised by the signs of depression, weakness, anorexia, ruffled feathers and a very easily recognizable clinical sign of bloody diarrhea. Mortality among the infected birds is highest between the sixth and eight day of infection owing to the excessive loss of blood. In the present study, caeca distended with

Table 3: Liver function test in the uninfected and infected chicken with *E. tenella*

Parameters	Methods	Normal value	Test sample
Glucose (mg/dl)	GOD-PAP	130-270	939.95
Cholesterol (mg/dl)	CHOD-PAP	125-200	50.95
Albumin (gm/dl)	Bromocresol green	2.3-3.3	1.82
Alanine aminotransferase (U/dl)	Reitman and Frankel	4.42	112.50
Aspartate aminotransferase (U/dl)	Reitman and Frankel	33.0	260.00
Alkaline phosphatase (U/L)	Mod. King and King`s	482.5	114.96

Table 4: Kidney function test in the uninfected and infected chicken with *E. tenella*

Parameters	Methods	Normal value	Test sample
Uric acid (mg/dl)	Uricase-PAP	1-2	42.73
Chloride (mmol/dl)	Thiocyanate-Hg	11.9-13.0	118.00
Magnesium (mg/dl)	Calmagite	2.2-3.2	11.18
Phosphorus (mg/dl)	Phosphomolybdate	3.6-7.2	7.82

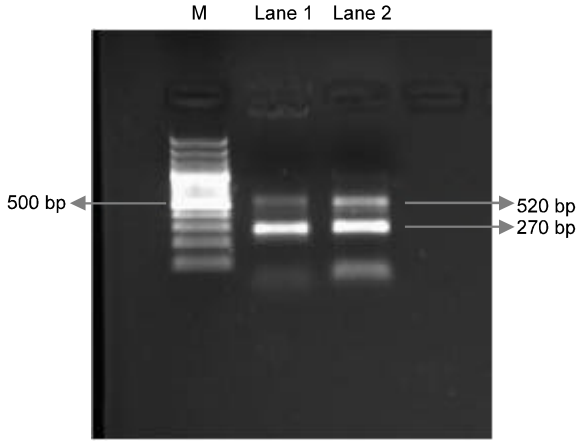

Fig. 3: Agarose gel electrophoresis of the PCR products. Lane 1 and Lane 2: Amplified PCR products obtained using both genus and *E. tenella* specific primers. Lane M: 100 bp marker

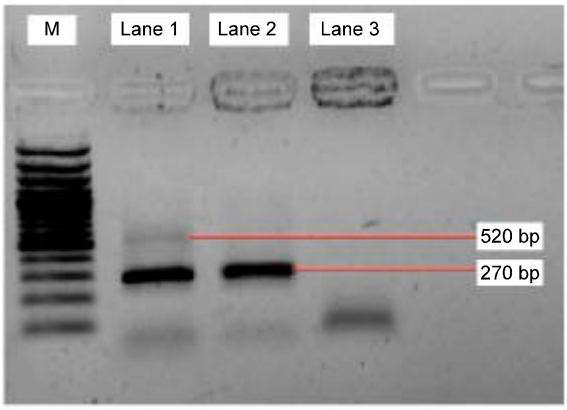

Fig. 4: Agarose gel electrophoresis of the PCR products. Lane 1: Amplified PCR product obtained using mixture of genus and species specific primers. Lane 2: Amplified PCR product obtained using *E. tenella* specific primer. Lane M: 100 bp marker

bloody faeces and mucoid debris along with haemorrhages on the mucosa was observed. The findings of this characteristic feature of caecal coccidiosis is in agreement to the report of Long (1973) who observed enlargement of caecum with clotted blood, haemorrhages throughout caecal mucosa, thickened caeca, shortened and consistent cases of necrosis and change in coloration from reddish to milky white.

Histopathological lesions observed (Fig. 1 and 2) in the present investigation are similar to the findings of Rasheda and Bano (1985). They also observed oedema and erosion of the sub-mucosa, glandular tissue cells infiltrations, in addition to caeseous necrosis and fatty decomposition.

The reduction in Hb and TEC may be attributed to haemorrhages in the caeca followed by development of caecal lesions. There may be liberation of large quantities of histamine due to injury to the tissues which increase the permeability of capillaries and venules allowing exudation of large quantities of fluid. Hein (1971) observed significant reduction in PCV on day 5 post infection in 2 week old chicken while Turk (1985) recorded fall in PCV from the day 5 to day 10 day with *E. necatrix* infection. The presence of macrocytic hypochromic anaemia in caecal coccidiosis infected birds was revealed by low levels of MCV and MCHC. The increase in lymphocyte count might be due to induction of immune response in the infected birds due to increased lymphopoiesis as a first step of defense mechanism to infection.

The significantly higher serum uric acid, Pi, magnesium and chloride levels in infected birds might be due to severe kidney dysfunction, metabolic acidosis, as well as intravascular haemolysis.

The high level of blood glucose observed in coccidia infected birds may be due to stress resulting in liberation of adrenal cortisones/corticosteroids which induce hyperglycaemia or disturbed carbohydrate metabolism with interference with phosphorylative carbohydrate dissimilation by unidentified material

present in intestine, as suggested by Daugherty and Herrick (1952). Marked alteration in intestinal physiology resulting in impaired carbohydrate absorption in acute phase of coccidiosis has also been reported previously (Turk *et al*., 1977). Waxler (1941) and Pratt (1940; 1941) have reported that chickens infected with *E. tenella* develop hyperglycaemia and there is a decrease in the amount of stored glycogen. Gessler (1965) reported that serum level of transaminases is affected by reduced feed intake. In the present study, the birds lost appetite during the peak of infection and this might have affected the transaminase level.

Alkaline phosphatase level was significantly reduced in the affected birds which is indicative of damages to the bone marrow with severe growth depression as ALP is known to rise during active growth (Reddy *et al*., 1988a; Reddy *et al*., 1988b; Nagalakshmi *et al*., 1996).

The reduced cholesterol level may be attributed to damage of liver and intestine by *E. tenella* leading to failure of its synthesis by the liver and intestine (Machado, 2002). *Eimeria* species were only unambiguously identified by PCR. Using the PCR assay *E. tenella* was found to be the only species present in the faecal sample of naturally infected birds. In the present investigation, interspecific DNA sequence diversity within the ITS1 region of rDNA from four pathogenic species of *Eimeria* was examined to reveal species specific DNA sequences applicable for diagnostic use. The fact that ITS1 region are less conserved than the rRNA genes, showing variations in DNA sequence and length, makes the design of primers straight forward and reduces the risk of cross reactions among different species (Holmdahl and Mattsson, 1996).

In this study, the specificity of PCR assay was assessed by positive amplification of DNA from *E. tenella* with species specific primer. No cross amplification by the other species specific primer were observed. Thus, the result of this study clearly demonstrates that the PCR assay based on amplification of ITS1 regions of *Eimeria* species in poultry can be used for detection and identification of the parasite. Further work is however needed to identify ITS1 primers for *E. maxima*, *E. mitis* and *E. praecox*, so that all seven species may be identified. This PCR technique, in comparison to conventional methods, is much faster and technically easier to use. It has a much higher sensitivity being able to detect the presence of only few parasites (Erlich *et al*., 1991; Persing, 1991). Although each test on template requires a separate reaction for each pair of primer, they can be run concurrently in the same PCR machine due to almost uniform annealing temperature of the primers.

REFERENCES

Arakawa, A. and M.Q. Xie, 1993. Control of coccidiosis in chicken. J. Protozoological Res., 3: 31-39.

Beate, E.S., L.T. Per, G.M. Jens, M.T. Fiona and W.S. Martin, 1998. Development of diagnostic PCR assay for the detection and discrimination of four pathogenic *Eimeria* species of the chicken. Avian Path., 27: 490-497.

Burg, J.L., C.M. Grover, P. Pouletty and J.C. Boothroyd, 1989. Direct and sensitive detection of a pathogenic protozoan, *Toxoplasma gondii* by polymerase chain reaction. J. Cl. Microbiol., 27: 1787-1792.

Cai, J., M.D. Collins, V. Mc Donald and D.E. Thompson, 1992. PCR cloning and nucleotide sequence determination of the 18S rRNA genes and internal transcribed spacer 1 of protozoan parasites *Cryptosporidium parvum* and *Cryptosporidium muris*. Biochimica et Biophysica Acta., 1131: 317-320.

Calnek, B.W., H.J. Barnes, C.W. Beard, L.R. Mc Dougald and Y.M. Saif, 1997. Disease of poultry. 10th Edn., Mosby Woife, United States of America.

Daugherty, J.W. and C.A. Herrick, 1952. Cecal Coccidiosis and carbohydrate metabolism in chickens. J. Parasitol., 38: 298-304.

Devereux, J., P. Haberli and O. Smithies, 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acid Res., 12: 387-395.

Erlich, H.A., D. Gelfand and J.J. Srinsky, 1991. Recent advances in the polymerase chain reaction. Science, 252: 1643-1651.

Gessler, M., 1965. Factors affecting changes in enzyme content in the blood of fowls. Zenbtl fur Vet. Med., 12A: 471-478.

Guay, J.M., D. Dubois, M.J. Morency, S. Gagnon, J. Mercier and R.C. Levesque, 1993. Dertection of th payhogenic parasite *Toxoplasma gondii* by specific amplification of ribosomal sequence using comultiplex polymerase chain reaction. J. Cl. Microbiol., 31: 203-207.

Hein, H., 1971. Pathogenic effects of *Eimeria necatrix* in young chicken. Exp. Parasitol., 65: 321-330.

Holmdahl, O.J.M. and J.G. Mattsson, 1996. Rapid and sensitive indentification of *Neospora caninum* by *in vitro* amplification of the internal transcribed spacer1. Parasitology, 112: 177-182.

Jeffries, A.C., N. Amaro, A.M. Tenter and A.M. Johnson, 1996. Genetic diversity in Sarcocystis gigantean assessed by RFLP analysis of the ITS1 region. Applied Parasitol., 37: 275-283.

Joyner, L.P. and P.L. Long, 1974. The specific characters of *Eimeria*, with special reference to the coccidian of the fowl. Avian Path., 3: 145-157.

Kawahara, F., K. Taira, S. Nagai, H. Onaga, M. Onuma and T. Nunoya, 2008. Detection of five avian *Eimeria* species by species-specific real-time polymerase chain reaction assay. Avian Dis., 52: 652-656.

Lew, A.E., G.R. Anderson, C.M. Minchin, P.J. Jeston and W.K. Jorgensen, 2003. Inter- and intra strain variation and PCR detection of the internal transcribed spacer 1 (ITS-1) sequences of Australian isolates of *Eimeria* species from chickens. Vet. Parasitol., 112: 33-50.

Lillie, R.D., 1965. Histopathologic Technique and Practical Histochemistry. 3rd Edn., Mc. Graw-Hill, New York, pp: 525-558.

Long, P.L., 1973. Pathology and pathogeneicity of coccidial infection. University Park Press, Baltimore, Maryland, pp: 251-294.

Machado, C.M., 2002. Crescimendo do TecidoAdiposo. In: Macari, M., Surlam, R.L., Gonzales, E., Fisiologia Aviara aplicada a frangos de corte. Jaboticabal: FUNEP-UNESP: pp: 375.

Muller, N., V. Zimmermann, B. Hentrich and G. Gotterstain, 1996. Diagnosis of *Neospora canninum* and *Toxoplasma gondii* by PCR and DNA, hybridization immuno assay. J. Cl. Microbiol., 34: 2850-2852.

Nagalakshmi, D., V.R.B. Sastry, D.K. Agrawal, R.C. Ketiyar and S.V.S. Verman, 1996. Performance of broiler chicks fed on alkali-treated neem (*Azadirachta indica*) kernel cake as a protein supplement. Por. Poult. Sci., 37: 809-818.

Paynee, S. and J. Ellis, 1996. Detection of *Neospora caninum* DNA by polymerase chain reaction. Int. J. Parasitol., 26: 347-351.

Persing, D.H., 1991. Polymerase chain reaction: trenches to benches. J. Cl. Microbiol., 29: 1281-1285.

Pratt, I., 1940. The effect of *Eimeria tenella* (Coccidia) upon the blood sugar of the chicken. Trans. Am. Microsc. Soc., 59: 31-37.

Pratt, I., 1941. The effect of *Eimeria tenella* (Coccidia) upon the glycogen stores of the chicken. Am. J. Hyg., 34: 54-61.

Quarzane, M., M. Labbe and P. Pery, 1998. *Eimeria tenella*: Cloning and characterizarion of cDNA encoding a S3a ribosomal protein. Gene, 225: 125-130.

Rasheda, M. and L. Bano, 1985. Histopathology of coccidiosis by *Eimeria garnhami* in *Cortunix cortunix* of N.W.F.P. Pak. Vet. J., 1: 27-29.

Reddy, V.R., P.V. Rao and C.V. Reddy, 1988a. Utilization of chemically neem oil in broiler chicks. India J. Anim. Sci., 58: 830-834.

Reddy, V.R., P.V. Rao and C.V. Reddy, 1988b. Chemical composition and nutritive value of variously processed neem cake. India J. Anim. Sci., 58: 870-874.

Reece, K.S., M.E. Siddal, E.M. Burreson and J.E. Graves, 1997. Phytogenetic analysis of Perkinsus based on actin gene sequences. J. Parasitol., 83: 417-423.

Sambrook, J.Z., E. Fritsch and T. Maniatis, 1989. Molecular Cloning, A Laboratory Mannual, 2nd edn. Cold Spring Harbor Laboratory Press, New York.

Shirley, M.W., 1995. *Eimeria* species and strain of chicken. In: Eckert, J., Braun, R., Shirley, M.W., Coudert, P. (Eds.). Biotechnology-Guidelines on techniques in coccidiosis research. European commission DG XII, Luxembourg, pp: 1-24.

Shirley, M.W., 1999. Coccidial parasites from the chicken. Their control by vaccination and some new tools to examine their epidemiology. In: International conference and exhibition of Veterinary Poultry Proceedings, pp: 44-60. Beijing, China.

Su, Y.C., A.C. Fei and F.M. Tsai, 2003. Diffential diagnosis of five avian *Eimeria* species by polymerase chain reaction using primers derived from the Internal Transcribed Spacer-1 (ITS-1) sequence. Vet. Parasitol., 117: 221-227.

Turk, D.E., P.L. Long, K.N. Boorman and B.M. Freeman, 1977. The effect of coccidiosis on intestinal function and gut microflora. Avian coccidiosis Proc. 13th Poult. Sci. Symp., 14-16, 227-267.

Turk, D.E., 1985. Macro-elements in the circulation of coccidiosis infected chicks. Poult. Sci., 65: 462-468.

Waxler, S.H., 1941. Changes occurring in the blood and tissue of chickens during coccidiosis and artificial haemorrhage. Am. J. Physiol., 134: 19-29.

Witlock, D.R. and M.D. Ruff, 1997. Comparision of the intestinal surface damaged caused by *Eimeria mivati, E. necatrix, E.maxima, E. brunetti* and *E. acervulina* by scanning electron microscopy. J. Parasitol., 63: 193-199.

Yadav, A. and S.K. Gupta, 2001. Study of resistance against some ionophores in *Eimeria tenella* field strains. Vet. Parasitol., 122: 69-75.