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Molecular Diagnosis of Naturally Infection with *Eimeria nieschulzi* in Laboratory Rats

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ABSTRACT

An outbreak of coccidiosis in laboratory rats has been described in this study. Clinically, out of 50 rats, 30 were died after showing symptoms of anorexia, emaciation and diarrhea. Post mortem examination showed the large and small intestine distended with necrotic and sloughed off mucosal epithelial cells with intestinal content. The examination of the gut contents revealed numerous *Eimeria* oocysts. After sporulation, the oocysts were identified as those of *Eimeria nieschulzi*. The infection due to *Eimeria nieschulzi* is confirmed by Polymerase Chain Reaction (PCR) after amplifying partial (~1.6 kb) nuclear 18S rDNA from DNA of *E. nieschulzi*. The histopathological examination showed severe damage of the lamina propria of the intestinal mucosa with numerous coccidian developmental stages in the epithelium of small intestine. Biochemical analysis of serum from infested rats presented a significant increase in serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST), glucose, total proteins, globulin and inorganic phosphorous while a decrease in alkaline phosphatase and blood urea nitrogen with insignificant changes in potassium, sodium, magnesium and chloride.

Key words: Coccidiosis, *Eimeria nieschulzi*, laboratory rats, biochemical profile, molecular diagnosis

INTRODUCTION

Coccidia are intracellular, obligate protozoa belonging to the phylum- Apicomplexa and are found in a wide variety of wild and domestic vertebrates (Duszynski and Upton, 2001). Coccidiosis affects the living host in many ways, depending upon the tissue preference of the particular parasite involved and the number of oocysts in the initial infection. Most of these parasites attack the mucosa of intestinal tract; therefore, symptoms are predominantly enteric. Two genera of

concern are *Eimeria* and *Isospora* (Van Kruningen, 1995). *Eimeria nieschulzi* is an intestinal coccidian uncommon in laboratory rats but is common in wild rats. Pathogenic effects of *E. nieschulzi* infection are usually seen in young rats less than six months of age. Signs include diarrhea, weakness, emaciation and possibly death. *Eimeria* species developing in the crypts of enterocytes or in the cells lamina propria mucosa might cause severe coccidiosis (Duszynski and Upton, 2001; Slapeta *et al.*, 2001). *Eimeria nieschulzi* is considered mildly pathogenic in small numbers. However, after primary infection with large number of sporulated oocysts, pathologic changes may be severe and include crypt hyperplasia, villus atrophy, increase villus to crypt ratio, cellular infiltration, oedema, hyperaemic to haemorrhagic bowel and increased mass of the small intestine. In addition to inducing pathologic lesions, *E. nieschulzi* infection reduces digestibility of dry matter, organic matter and nitrogen. Also, *E. nieschulzi* may alter the host response to concurrent or subsequent infection with other parasites (Al-Dahwi *et al.*, 2006). 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. The presence of parasites may be confirmed by post-mortem examination of the host or by fecal examination. Experimentally, passaging oocysts through chickens provide relevant information regarding prepatent periods and immunogenicity against reference strains (Shirley *et al.*, 2005). However, the accurate identification of parasites (irrespective of developmental stage and sex) and their genetic characterization have important implications for systematic (taxonomy and phylogeny, population genetics, ecology and epidemiology) and also centre to diagnosis, treatment and control of the disease they cause (Gasser and Chilton, 2001).

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; Su *et al.*, 2003; Lew *et al.*, 2003; Kawahara *et al.*, 2008). Although there are some reports of coccidial infection in rats (Sevinc, 2001), pathology and biochemical observation in rat coccidiosis is very rare. The current study aimed to analyze the histopathological changes in the small intestine of laboratory rats naturally infected with *E. nieschulzi* along with some selected biochemical parameter changes induced by the parasite.

MATERIALS AND METHODS

Of the 50 white albino outbred rats, of either sex and aged 3-4 months maintained in the college animal house, 15-20 rats started showing the symptoms of anorexia, diarrhea and weakness during the month of January, 2010. Within 3 days from the onset of these clinical symptoms, the total number of affected rats rose to 40 with mortality of 30 rats. Preliminary examination of faeces of the affected rats revealed presence of large number of unsporulated oocysts. In order to investigate and confirm the cause of mortality, faeces and blood were collected from a group of 10 rats each without clinical symptoms, with clinical symptoms and those died. Faeces were examined under microscope for coccidian oocysts and serum was separated and kept at -20°C until it was analysed for alanine amino transferees (ALT), aspartate amino transferees (AST), alkaline phosphatase (ALP), glucose, total proteins, albumin and globulin; blood urea nitrogen, inorganic magnesium, potassium, sodium, chloride and phosphorus using standard diagnostic kits available



Fig. 1: Tympanic changes in gut of laboratory rats

in the market (Coral Biosystem) as biochemical indicators for liver and kidney functions. The post mortem of dead rats were conducted, lesions present were recorded and a piece of intestine showing lesions were also taken from the representative rats for histopathology. Tissues collected were fixed in 10% formalin, embedded in paraffin, sections were cut at 5 μ m and stained with Haematoxylin and eosin (H&E). Pooled faecal samples were also collected for sporulation of oocysts in 2.5% potassium dichromate solution. Sporulated oocysts were then identified and were also used for molecular diagnosis.

DNA extraction: The purified oocysts, stored in 2.5% potassium dichromate solution, were washed 4 times by centrifugation (1.4×10^3 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 μ L 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 using ultrasonicator (OMNI Ruptor400, Made in USA) to disrupt the oocysts. The DNA was extracted from sonicated oocysts and analysed by agarose gel electrophoresis.

PCR amplification and identification of the PCR products: For testing nuclear DNA, the 18S rDNA was amplified using primers 18SF1, 5'-GCTTGTCTCAAAGATTAAGCC and 18SR2, 5'-AGCGACGGGCGGTGTGTACAA.

PCR: The PCR was done in Thermo Cycler C 1000 (BioRad, USA), with 50 μ L of reaction mixtures for each sample that contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM MgCl_2 , 200 μ M of each dNTPs, 100 pmol (25 pmol each of species specific oligonucleotide primer of *Eimeria nieschulzi* and target DNA in each tube. 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

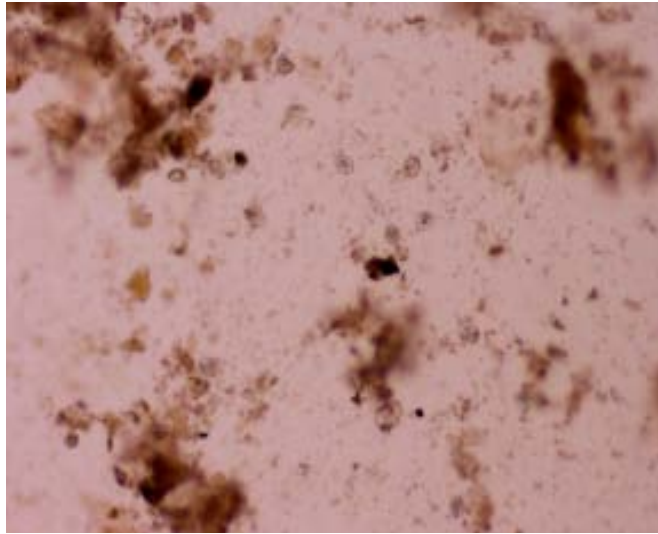


Fig. 2: Unsporulated oocysts of *Eimeria nieschulzi* (10x)



Fig. 3: Sporulated oocyst of *Eimeria nieschulzi* of rat (100x)

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).

RESULTS

Of 50 rats reared in the college animal house, 30 out of 40 died after showing symptoms of anorexia diarrhea and emaciation. The microscopical examination of faeces content showed numerous oocysts. The sporulated oocysts were ellipsoidal to ovoid, tapering both ends. Wall was smooth, yellowish and measured 22.51-14.77 μm (on average) with no micropyle and it was

Table 1: Serum biochemical profile of laboratory rats infected with *Eimeria nieschulzi*

Parameters range	Methods	Test Sera	Normal
Glucose (mg dL ⁻¹)	GOD-PAP	216.86	47.7-107.0
Total Protein (g dL ⁻¹)	Biuret	10.60	7.5 ± 2.7
Albumin (g dL ⁻¹)	Bromocresol green	4.53	4.17 ± 2.1
Globulin (g dL ⁻¹)		6.03	3.33
Urea (mg dL ⁻¹)	Uricase-PAP	13.39	16.9 ± 2.1
Magnesium (mg dL ⁻¹)	Calmagite	3.61	3.12
Potassium (mmol L ⁻¹)	Colorimetric	8.76	6.50±1.33
Sodium (mmol L ⁻¹)	Colorimetric	141.49	146.8±0.93
Chloride (mmol L ⁻¹)	Thiocyanate-Hg	93.51	79.44-111.3
Phosphorus (mg dL ⁻¹)	Phosphomolybdate	8.38	2.29±0.38
Alanine amino transferase (ALT) (U L ⁻¹)	Reitman and Frankel	182.60	35.1±13.3
Aspartate amino transferase (AST) (U L ⁻¹)	Reitman and Frankel	455.90	42.9±16.1
Alkaline phosphatase (ALP) (U L ⁻¹)	Mod. King and King's	10.36	133±13.4



Fig. 4: Intestinal mucosa showing cellular infiltration, sloughing of mucosa and developmental stages of *Eimeria nieschulzi* (10x)

identified as *Eimeria nieschulzi* (Fig. 2, 3). In histopathological sections, numerous coccidian developmental stages were seen in the epithelial cells. The histopathology included substantial hyperplasia of the crypt enterocytes, damage to the surface mucosa and extensive infiltration of the mononuclear cells in the lamina propria with sloughing of the mucosal epithelium (Fig. 4, 5). Based on clinical symptoms, substantiated by faecal examination and histopathological changes, it was suggesting coccidiosis to be the cause of mortality caused by *Eimeria nieschulzi*.

Biochemical findings: Liver function test of infested rats showed a significant increased in alanine amino transferase (ALT) and aspartate amino transferase (AST) while the levels of serum urea and alkaline phosphatase (ALP) were decreased with variation in the level of glucose, total protein, globulin and inorganic phosphorus (Table 1).

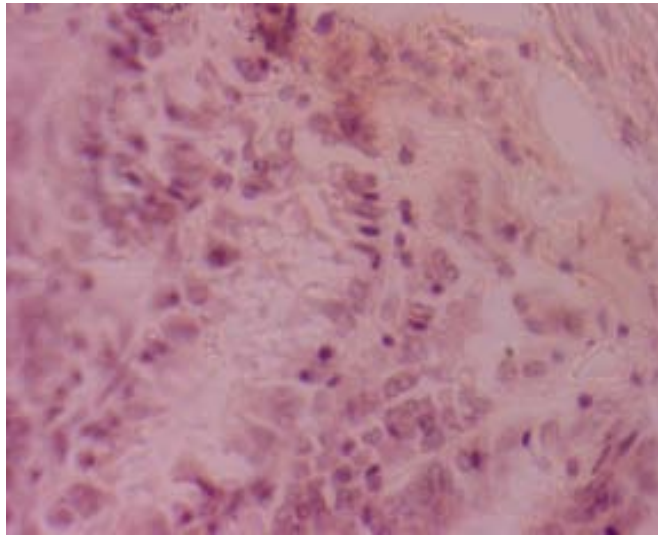


Fig. 5: Intestinal mucosa showing cellular infiltration, sloughing of mucosa and developmental stages of *Eimeria nieschulzi* (40x)

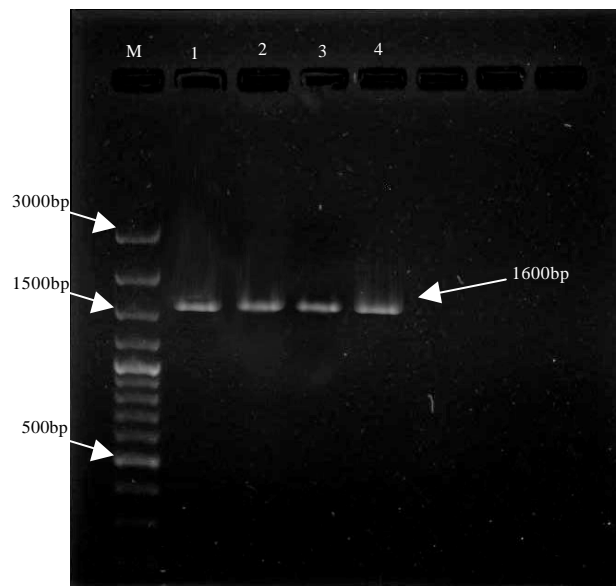


Fig. 6: PCR results of partial (~1.6 kb) nuclear 18S rDNA amplified from DNA of *E. nieschulzi*

PCR amplification: The primer pair for nuclear DNA specific for nuclear 18S rDNA of *E. nieschulzi* exclusively amplified their target DNA as shown in Fig. 6.

DISCUSSION

Coccidiosis is caused by a parasitic protozoan, which lives in the gut. Coccidia are obligate intracellular parasites whose development within the cytoplasm of epithelial cells results in the

death of parasitized cell. When many cells of the intestinal epithelium are parasitized at one time, the denuded mucosa may bleed freely and intense inflammation involves the lamina propria and sometimes the submucosa. As large numbers of epithelial cells are destroyed the remaining epithelium is stimulated to replace that which was lost in domestic animals (Jones *et al.*, 1997). There is little knowledge about morphology, histopathology of *Eimeria* sp. that caused coccidiosis in laboratory rats. Rose and Hesketh (1982) investigated infection with *Eimeria nieschulzi* and noted the changes in increase in gut weight, partial villous atrophy and increased numbers of mast, goblet and pyroninophilic cells. Blagburn and Todd (1984) observed histopathologic changes consisting of an early neutrophil and mononuclear cell infiltration in the small intestine and later, villus atrophy and crypt hyperplasia caused a decrease in the villus-crypt ratio in experimental *Eimeria vermiformis* infections in *Mus musculus*. These findings are similar to the observation made in the present investigation.

In the present study, high level of blood glucose has been observed in naturally infested rats with *Eimeria niestulzi*. The present findings on blood glucose are in agreement with those of Uday Kumar and Padmavathi (2000) and Hirani *et al.* (2007). The high level of blood glucose observed in coccidian infected rats may be either due to stress condition leading to the 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 in chickens infected with *Eimeria tenella*, hyperglycaemia develops, and there is a decrease in the amount of stored glycogen. Stein and Marquardt (1973) observed increased uptake of glucose into small intestinal tissue and hepatic portal plasma at 3 days postinoculation and decreased absorption at 8 days postinfection with experimental infection with *Eimeria nieschulzi* compared to uninfected control rats.

An increased in level of the total serum protein and serum globulin without a marked change in the level of serum albumin was observed in the present investigation. The reason for high level of serum total protein and globulin in the serum profiles of infested rats is due to infection by the *Eimeria niestulzi*. Clinico-pathologic changes in natural and/or experimental infection in rabbits with *Eimeria stiedai* reported by various workers (Abdel Ghaffar *et al.*, 1990; Barriga and Arnoni, 1979, 1981; Pakes and Gerrity, 1994) and these changes include increase in β and γ globulin, β -lipoprotein, succinate dehydrogenase (which later declines), bilirubin, alanine transaminase and aspartate transaminase levels in serum. The present finding is in contrast to the findings of Stockdale *et al.* (1981). Stockdale *et al.* (1981) observed mild decrease in mean total plasma protein and mean plasma albumin levels in the calves experimentally infected with *E. zuernii*. Decreases in α -lipoprotein, glucose and protein levels in serum and in alkaline phosphatase activity in the liver have also been reported. The increase in the level of serum enzyme activity of ALT and AST in the present investigation is similar with the findings of Cam *et al.* (2008) in their experimental study of rabbits with *Eimeria stiedai*. A decreased in the level of serum alkaline phosphatase activity was observed in the present investigation. The decreased level of serum alkaline phosphatase in the present study is in agreement with the observations made by Abdel Ghaffar *et al.* (1990), Barriga and Arnoni (1979), Barriga (1981) but is in contrast to the finding of Manning *et al.* (1994) where they found an increase level of ALP in young rabbits (2 months old) infected with hepatic coccidiosis. Reddy *et al.* (1988) and Nagalakshmi *et al.* (1996)

reported that Alkaline phosphatase level was significantly reduced in the birds infected by coccidia which is indicative of damages to the bone marrow with severe growth depression as ALP is known to rise during active growth. Similarly, there may be damage to the bone marrow of the coccidia infected rates which results in the decrease activity of the alkaline phosphatase.

Serum analysis also showed slight decreased level of sodium and increased level of potassium. This electrolyte disturbance can be attributed to diarrhea. The present finding is in agreement with the findings of Fitzgerald (1967), who noted only a minor changes in the serum sodium and potassium level in the calves infected with *E. bovis*. The serum sodium level decreases slightly while the level of potassium marginally increases until the infection is severe. He also noted an increase in serum potassium in severely infected calves. Veli *et al.* (2004) analyzed coccidian related alterations in intestinal function by ion transport and electrophysiological investigation in rats experimentally infected with *Eimeria separata* for possible cause of diarrhea due to coccidiosis.

From the present observations, we can conclude that the outbreak of coccidiosis may cause severe problems in the laboratory rats even under modern condition of breeding and maintenance.

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