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ELISA Assessment in the Diagnosis of Hepatic Coccidiosis in Experimentally Infected Rabbits

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Abstract: The present study aims to early diagnosis of hepatic coccidiosis during the course of experimental infection of rabbits with *Eimeria stiedae*. Oocyst counts, histopathology and ELISA were used as diagnostic tools. ELISA using oocysts antigen can detect *E. stiedae* antibodies at first week after infection and reached its highest level 21st day post infection (PI). While, shedding of oocysts began from day 16th and reached the highest oocyst counts (276860 oocysts /g) 22nd day PI, then began to decline till 1000 oocysts/g 46th day post infection. Oocysts were not detected in feces from day 47th till the end of the experiment. Histopathological examination showed hyperplasia of the bile duct epithelium with different developmental stages of coccidia oocysts in the lumen. Granuloma tissues encircled the bile duct with infiltration of inflammatory cells. Gross necropsy finding, hepatomegaly and multiple scattered yellowish white nodules of variable sizes throughout the liver were observed. The gall bladder may also be enlarged and contain exudates. In conclusion, ELISA using oocyts antigen proved to be the best tool for early diagnosis of hepatic coccidiosis and can be used in field studies in order to assess coccidiosis seroprevalence in rabbit farms.

Key words: *Eimeria stiedae* · Hepatic Coccidiosis · Histopathology · Rabbits · ELISA

INTRODUCTION

Coccidia are one of the most important groups of protozoa that affect many animal and avian species [1]. *Eimeria stiedae* is one of the most pathogenic *Eimeria* species of domestic rabbits. *E. stiedae* parasitizes the epithelial cells of the bile duct causing severe liver damage and great economic losses [2, 3]. It causes hepatomegaly, hypertrophy and hyperplasia of the bile ducts, Periportal fibrosis and disorganization of the hepatic parenchyma leading to ascitis [4]. Its diagnosis is mostly based on postmortem examination of the liver or on coprological assays for *in vivo* diagnosis [5]. However, these methods have scant sensitivity and only permit late diagnosis. Alternatively, serodiagnosis provides advantage in accurate and early detection of parasite infection in animals [6,7]. Immunodiagnosis of hepatic coccidiosis was previously probed using different antigens [8-10].In addition, ELISA exhibited good performance in the detection of antibodies to *Cyclospora cayetanensis* in human sera using a combination of *Eimeria tenella* and *Eimeria zurnii* antigens [11]. Furthermore, ELISA using *Eimeria tenella* merozoite and sporozoite antigens should prove useful for monitoring infectivity in vaccination programs in layer and breeder flocks and for assessing the effectiveness of biosecurity measures in broiler flocks [12]. In addition, antibody responses elicited by infections with wild-type and attenuated strains of *E. tenella* and *E.necatrix* were characterized by immunoblotting and ELISA with homologous and heterologous antisera [13].They found that in ELISA conducted with merozoite antigen preparations, antiserum from birds infected with the wild-type strains of *E. tenella* and *E. necatrix* consistently produced a significantly higher ($P<0.05$) antibody response than antiserum from birds infected with the attenuated strains.

The present study was designed to diagnose hepatic coccidiosis in rabbits by parasitological, histopathological and serological examinations. Also, the detection of the most appropriate method for early diagnosis of the disease is an important objective.

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MATERIALS AND METHODS

Preparation of *E. stiedae* Sporulated Oocysts: The occysts of *E. stiedae* were collected from gall bladders and necrotic hepatic lesions of naturally infected rabbits. The livers and gall bladders were collected, minced and digested in 0.25% trypsin in normal saline. The digested materials were sieved, centrifuged at 2000 rpm for 10 minutes and washed several times by saline solution. The oocysts were counted as the method described by Ryley *et al*. [14] and identified according to Levine [15]. The oocysts were sporulated in 2.5% potassium dichromate. The sporulated oocysts were kept at 4°C until use for experimental infection and preparation of antigen.

Experimental Infection of Rabbits with *E. stiedae* Oocysts: A total number of 35 Newzealand white rabbits (5 weeks old and about 1.5-2 kg body weight) were used in the present study. Animal fecal samples were examined daily for 2 successive weeks to confirm that animals were free from coccidial oocysts. Rabbits were divided into 2groups.The first group was consisted of 25 rabbits, each was experimentally infected with 50000 sporulated oocysts and the second group (10 rabbits) was left as non infected control. All rabbits were examined for 7 weeks (the end of the experiment). Blood samples were weekly collected from infected rabbits. Fecal samples were collected from day 14th PI till the end of the experiment for determination of the number of *E. stiedae* oocysts per gram by the modified McMaster technique [16].

Histopathological Studies: Specimens were collected weekly from liver of infected rabbits. Specimens were fixed directly in 10% neutral formalin. The fixed specimens were embedded in paraffin wax to form tissue blocks. Sections from the blocks of 5 micron thickness were prepared and stained with haematoxylin-eosin "H and E" stain and examined microscopically [17].

Serological Studies

Rabbit Sera Collection: Serum samples were collected from blood of infected and control rabbits weekly from zero day till seven th week PI. Samples were stored at-20°c until use.

Preparation of Antigen: *E. stiedae* oocyst antigen was prepared according to Mousa *et al.* [18]. The oocysts were homogenized for 15 minutes on ice followed by sonication for 5 minutes. The homogenates were centrifuged at15, 000 rpm for 45 minutes at 4°c. The protein content of the supernatant was determined according to Lowry *et al.* [19]. The antigen was aliquoted and stored at-20 °c until use.

Enzyme Linked Immunosorbent Assay (ELISA): The diagnostic potency of *Eimeria* oocysts antigen was evaluated by ELISA which was performed according to Santiago *et al*. [20] with little modifications. The optimum antigen concentration, sera and conjugate dilutions were determined by checkerboard titration. The plate was coated with oocyst antigen in coating buffer. After coating, 100 µl from diluted infected or control sera were added to each well. Horse radish peroxidase-conjugated IgG anti-rabbit was used.Ortho-phenylene diamine was used as a substrate. The reaction was terminated with 1M H_2SO_4 and the absorbance values were read spectrophotometrically at 405 nm. The cut off value of optical density (OD) was calculated by method of Hillyer *et al.* [21].

RESULTS

Oocyst Counts: Shedding of oocysts began from day 16th and increased gradually. The highest oocysts count (276860 /g) was recorded 22 days PI and began to decline till day 46th which was 1000/g. Oocysts disappeared from feces starting day 47 th till the end of the experiment. No oocysts were detected in non infected rabbits (Table 1).

Table 1: Mean count of oocysts collected from rabbits experimentally infected with *E. stiedae* sporulated oocysts

	Mean oocysts count / gm	
Days post infection	Infected group	Non infected group
14	0	0
15	0	0
16	70230	0
17	94050	0
18	168200	0
19	170820	0
20	229150	0
21	235400	0
22	276860	0
23	251580	0
24	138500	0
25	121500	0
26	110260	0
27	105600	0
28	65200	0
29	56150	0
30	55200	0
34	30250	0
38	25250	0
42	15500	0
46	1,000	0
47	0	0

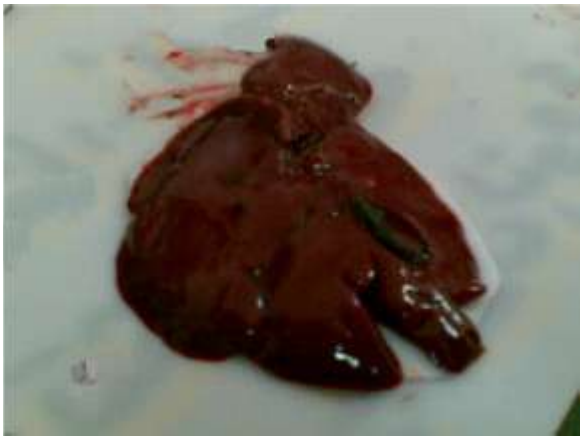

Fig. 1: Liver of infected rabbit with hepatic coccidiosis showing hepatomegally, pale color and multiple scattered yellowish white nodules of variable sizes

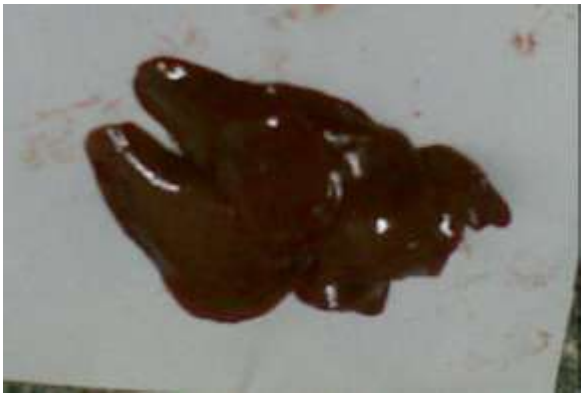

Fig. 2: Normal liver of healthy control group.

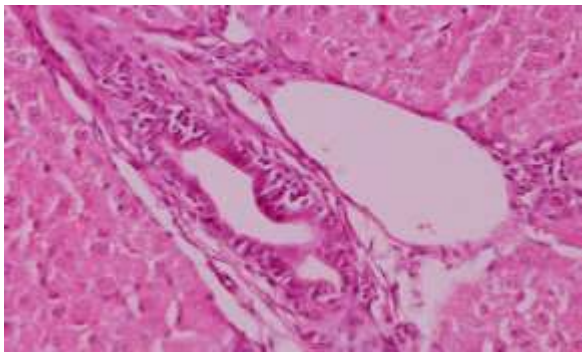

Fig. 3: T.S. in infected liver at day 7th PI showing hyperplastic epithelial lining of the bile ducts and variable degenerative changes that ranged from hydropic to vacuolar degeneration. H and E X400

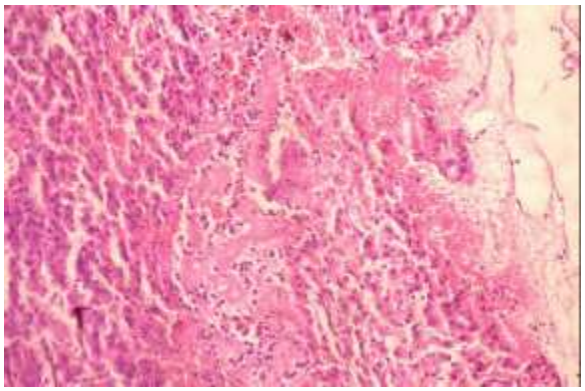

Fig. 4: T.S. in infected liver at day15th PI,showing mild hyperplasia of bile duct's epithelium with edema in the lamina propria and in submucosa. Multiple areas of degeneration of hepatic cells surrounded with inflammatory cells were observed.H and E X 200.

Pathological Changes

Macroscopic Lesions: The macroscopic picture of hepatic coccidiosis was enlargement of liver and pale color. Multiple scattered yellowish white nodules of variable sizes containing creamy fluid packed with oocysts were observed on both hepatic and cut surfaces. The gall bladders were enlarged and distended (Fig. 1) compared with non infected rabbits (Fig. 2).

Histopathological Changes: Examination of livers and gall bladders of experimentally infected rabbits showed moderate pathological changes. It was found from the 7th day onwards, the liver was enlarged and trophozoites were evident inside the hyperplastic epithelial cells lining bile ducts. The majority of the hepatic cells showed variable degenerative changes that ranged from hydropic to vacuolar degeneration (Fig. 3). Histopathological lesions in the liver at days 15th,21st showed sever congestion and dilatation of central veins, rupturing of the lining endothelial cells, hyperplasia of the lining epithelium of the portal areas, congestion and dilatation of sinusoids with hemorrhage areas. Multiple areas of degeneration of hepatic cells surrounded with inflammatory cells were observed. There was edema in the lamina propria and in submucosa; schizogony and gametogony (Fig. 4, 5)) beside the formation of the oocysts (21days) inside the hyperplastic epithelial lining of the bile ducts (Fig. 5). Microscopical examination at day28th showed dilatation of bile ducts with epithelial hyperplastic features which displayed fingerlike extension into the lumen. Various developmental stages of *E. stiedae* were observed in the epithelial cells of the ducts. There were fibrous tissue formation and inflammatory cell infiltration at the periphery of these ducts (Fig. 6).

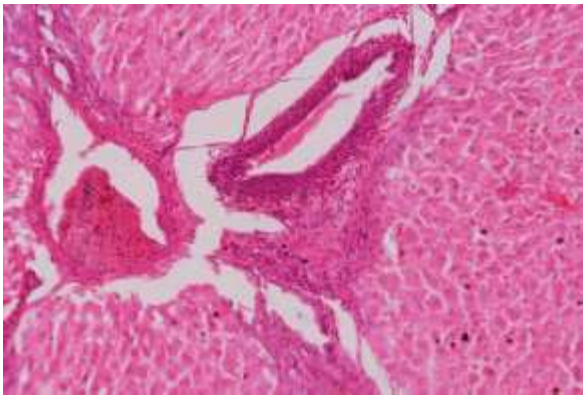

Fig. 5: T.S. in infected liver at day 21st PI,showing sever congestion and hyperplasia of bile duct's epithelium with proliferation of fibrous connective tissue around it. H&EX 200

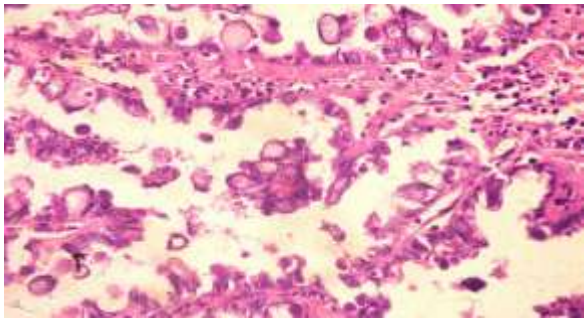

Fig. 6: T.S. in infected liver at day 28th PI,showing hyperplasia of bile duct's epithelium forming finger like projection, gametes of coccidia in the epithelium and in the lumen of bile ducts and inflammatory cells infiltration. H&EX400.

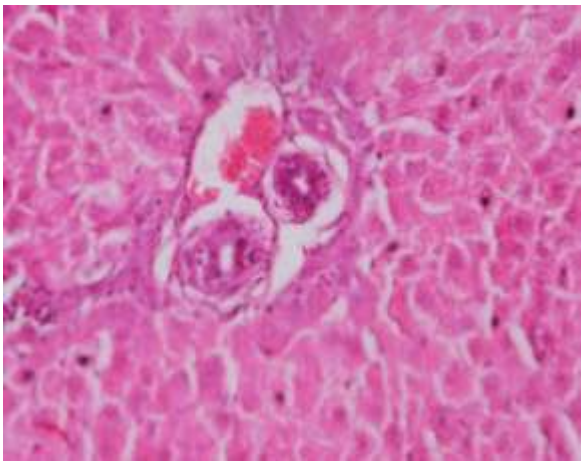

Fig. 7: T.S. in infected liver at day 35th PI., showing dilatation of bile ducts and extensive proliferation of the billiary epithelium. Schizonts and oocysts of E. stiedae were observed in the epithelial cells of the bile ducts. H and EX200.

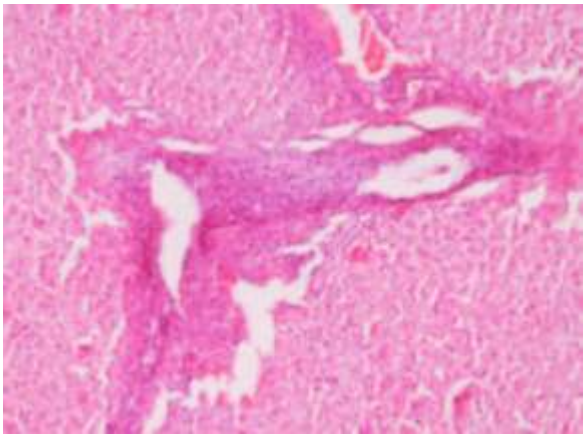

Fig. 8: T.S. in infected liver at day 42nd PI, showing focal necrotic area and granuloma tissues encircled the bile duct with infiltration of inflammatory cells H and EX200.

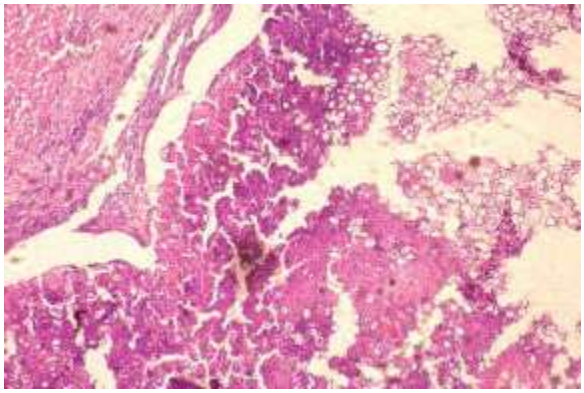

Fig. 9: T.S. in infected liver at day 49 PI, showing extensive hyperplasia of the ductular epithelium. Developmental forms of the parasite were seen in the bile duct epithelial cells and oocysts appear in the lumen. H and E X 200

The hepatocytes at days 35 th and 42nd showed focal areas of necrosis infiltrated by inflammatory cells and hemorrhagic areas. The bile ducts were dilated and there was extensive proliferation of the billiary epithelium. Schizonts and oocysts of *E. stiedae* were observed in the epithelial cells of the bile ducts. Oocysts could be seen in the lumen and granuloma tissues encircled the bile duct with infiltration of inflammatory cells. (Fig. 7,8). At day 49th the nodules were consisted of dilation and thickening of the bile duct due to extensive hyperplasia of the ductular epithelium. Developmental forms of the parasite were seen in the bile duct epithelial cells and oocysts appeared in the lumen. The liver parenchyma was destroyed by pressure from expanding biliary ducts and was gradually focally replaced by proliferated fibrous tissue infiltrated

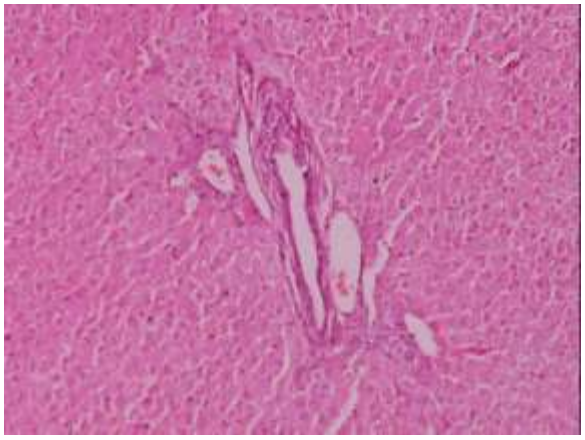

Fig. 10: T.S.in liver of non infected rabbit showed normal bile ducts with abundant normal hepatocytes. H and E X 200.

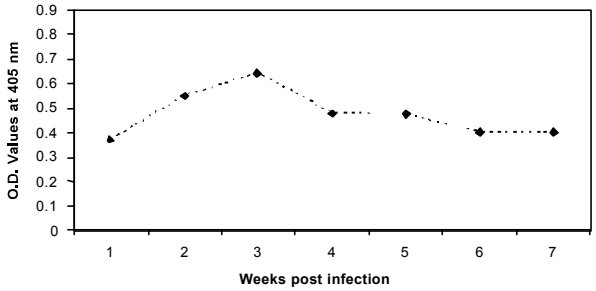

Fig. 11: Antibodies level in sera of rabbits experimentally infected with *E. stiedae* oocysts

with lymphocytes, macrophages, plasma cells and eosinophils (Fig. 9). Normal liver cells and bile duct epithelium of non infected group were shown in Fig.10.

Serodiagnosis of Hepatic Coccidiosis: The results of serological diagnosis of hepatic coccidiosis by ELISA showed increase in antibody level from the first week PI and reached the highest level at third week (Fig 11).The antibody level dropped at fourth week, then declined slightly before reaching a plateau at 6-7 weeks PI. No antibodies were detected in control negative rabbit sera.

DISCUSSION

In the current research, parasitological diagnosis of *E. stiedae* in experimentally infected rabbits revealed that the beginning of oocysts shedding was at the 16th day post infection.Simmilar results were detected by kutkat *et al.* [8] and Abdel-Megeed and Abu-El Ezz [22]. In addition, comparable results were observed by Gomez-Bautista *et al.* [23] and Sanyal and Sharma [24] who found that the first gamonts appeared on the 14 th-15 th days post infestation. In contrary, [25] reported that the first oocystic shedding appeared at the 3rd-4 th week post-infection.

The highest oocyst count per gram feces was observed at the 22nd day after infection. This results are matched with Kutkat *et al.* [8] and Abdel-Megeed and Abu-El Ezz [22] and comparable to that of Abdel-Megeed *et al*. [26] who proved that highest oocyst count was observed at 21 day post infection.While, [27, 28] observed that the highest oocyst concentration was seen between the 17th-21st days post infection.The highest oocysts count was 276860 / g. The lowest opg value was 1000 /g at day 46th. Oocysts disappeared in feces from day 47 th till the end of the experiment. Different highest oocysts numbers were reported by different authors, 251,750 opg [22], 50,300 opg [8] and 1,121,000 opg [29]. This difference in the oocysts count might be due to *E. stiedae* oocysts infection dose and age susceptibility.

In the present study, macroscopic examination of experimentally infected liver showed hepatomegally, pale color and multiple scattered yellowish white nodules of variable sizes. On squeezing the nodules, creamy caseous matter exudates with numerous oocystic zygotes and epithelial debris were observed microscopically. Present obtained results matched with those obtained by Barriga and Arnoni [30] who suggested the occurrence of those coccidian nodules due to toxic effect of protozoon which settled in the liver.

In the present study, gall bladders of infected rabbits were enlarged and distended, bile ducts were dilated and contained yellowish exudates. There are characteristic lesions of *E. stiedae* infection, these lesions may be due to intense biliary hyperplasia and fibrosis and their number might be taken as a criterion for assessing the degree of infection. These observations were agreement with Patrick *et al*. [31-33, 28].

Microscopically, bile ducts were dilated and there was extensive proliferation of the billiary epithelium. Various developmental stages of *E.stiedae* were noticed in the biliary epithelium. Oocysts could be seen in the lumen and granuloma tissues encircled the bile duct with infiltration of inflammatory cells. Hepatic cells suffered from damage, cloudy swelling and hydropic degeneration. Similar results were observed by Cam *et al*. [33] who suggested that *E. stiedae* infection causes lipid peroxidation resulting from the destruction of the bile duct and subsequently the hepatic parenchyma. This peroxidation of cell membrane causes impairment in its permeability and triggers a series of reactions that may result in cell death and lead to liver fibrosis [34] which concided with present data.

In the present study, using *E. stiedae* oocyst antigen in ELISA, IgG antibody response in experimentally infected rabbits started from 7 day post-infection and reached its maximum level at third week, then declined slightly before reaching a plateau and persisting at a high level for the duration of the experiment. Comparable results were obtained by Kandil *et al.* [8] who reported that IgG antibody response reached its maximum level at18th day post infection. Also, these results were matched with Constantinoiu *et al*. [12] although they used merozoite and sporozoite of *E. tenella* antigen. In the current study it was of interest to mention that serological results confirmed those obtained from parasitological and histopathological examination.

CONCLUSION

ELISA based on *E. stiedae* oocysts antigen could be useful for performing faster and early diagnosis of hepatic coccidiosis and have great value in epidemiological studies and control of this economic disease.

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