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Original Article

Molecular diagnosis of *Eimeria stiedae* in hepatic tissue of experimentally infected rabbits in comparison with traditional methods

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Abstract:

The early detection of *Eimeria stiedae* stages in hepatic tissue of experimentally infected rabbits was studied. The experiment was conducted using 40 male New Zealand rabbits of six weeks age. The rabbits were divided into an infected group (A) of 30 rabbit and a control uninfected group (B) of ten rabbits. Group A was infected with 2.5×10^4 sporulated oocysts of *E. stiedae* per rabbit at zero day. Three rabbits of group A and one of group B were sacrificed at 0, 3, 6, 9, 12, 15,18,21,24 and 27 day post-infection. Gross findings, light microscopy, transmission electron microscopy (TEM) and molecularly with PCR were applied to detect specific findings of *E. stiedae* in the liver tissue pre- and post- shedding of oocysts in the feces. Grossly, liver showed irregular yellowish white nodules appeared from the 15th days post-infection and became more prominent gradually. Hepatomegaly and ascites were obvious from the 21-24th day post-infection. Histopathologically, different schizonts and gametocytes of *E. stiedae* in the biliary epithelium appeared at the 15th day post-infection. Findings of TEM were matched with the light microscopy. PCR showed positive findings starting from the 12th day post-infection using specific *E. stiedae* primers and its specific amplicon of *E. stiedae* (976 bp). The shedding of oocysts began from the 17th day post- infection and reached the peak at the 23-25th day PI, then began to decline until the end of the study. In conclusion, the convention PCR detected *Eimeria* schizont from the 12thday post- infection earlier to PM lesions or before shedding of the oocysts in feces.

Key words: *Eimeria stiedae,* rabbits, experimental infection, PCR

INTRODUCTION

Coccidiosis has a particular importance in rabbit colonies. It is a highly contagious protozoal infection. It is caused by *Eimeria* species which are highly host, organ and tissue specific **(Levine, 1985; Georgi and Georgi, 1990).** Rabbit coccidiosis always present in rabbits farms and its eradication is very hard **(Vancraeynest et al., 2008)**. Hepatic coccidiosis (*E. stiedae*) is one of the most pathogenic coccidian protozoans in domestic rabbits causing severe disease and a high mortality **(Hauptman et al., 2001; Al-Mathal, 2008)**.

Eimeria stiedae is an inhabitant of the epithelial cells of the bile ducts and cause severe liver damage in rabbits **(Yakhchali and Tehrani, 2007; Once et al., 2011; Abed and Yakoob, 2013).** It causes proliferation of bile duct epithelial cells and the affected livers contained multifocal, well-demarcated, linear, yellow lesions due to the course of the biliary tree **(Hobbs and Twigg, 1998)**. The use of the biotechnological advances gave a significant step to improve the prevention and control of coccidiosis in poultry farms **(Morris and Gasser, 2006**). **Schnitzler et al. (1999), Lew et al. (2003)** and **Su et al. (2003**) used ITS-1 as a target for PCR to identify chicken *Eimeria* species. **Oliveira et al. (2011)** developed a molecular assay to differentiate between 11 *Eimeria* species in rabbits. So, the present study tries to use the molecular assay to investigate *E. stiedae* course in hepatic tissue and blood of experimentally infected rabbits. Also, comparing it with traditional methods at different intervals of the life cycle in order to achieve the most accurate results for early detection of the disease and its follow up in the patent period.

MATERIALS AND METHODS

1. Rabbits

Forty male healthy New Zealand rabbits, aged 6 weeks and weighing 1-1.5 kg, were used. During the experiment, the rabbits were individually housed in metal cages with a metallic grid on the bottom keeping rabbits from coming in contact with their feces. The rabbits were fed with commercial pellet food and water was supplied *ad libitum*.

The absence of *E. stiedae* and other coccidian oocysts prior to the experiments was confirmed by fecal examination using floatation method daily for two successive weeks to confirm that rabbits are free from coccidian infection.

2. *E. stiedae* oocysts

Previous molecularly identified *E. stiedae* oocysts **(Hassan et al., 2014)** were allowed to sporulate in 2.5% potassium dichromate solution (≥ 3 days).

3. Experimental infection of Rabbits with *E. stiedae* oocysts

The rabbits were divided into two groups; group A of 30 infected rabbits and group B of 10 control uninfected rabbits. Each rabbit of group A was challenged with a dose of 2.5×10^3 sporulated oocysts of *E. stiedae.* Three rabbits of group A and one animal of group B were sacrificed at 0, 3, 6, 9, 12, 15,18,21,24 and 27 day post-infection.

***4. E. stiedae* cycle in the infected rabbits was investigated through the following parameters:**

4.1. Parasitologically:

4.1.1. Prepatent and patent period:

The fecal samples of each infected animal were collected daily and examined microscopically for the appearance and existence of coccidian oocysts. The prepatent and patent period were stated.

4.1.2. Oocyst shedding rate:

Fecal samples were collected daily for parasitological examination beginning from oocyst excretion in feces until the 27th day post challenge and number of oocysts per gram feces was counted using the McMaster method **(Long and Joyner, 1976).**

4.2. PM & Histopathological lesions:

PM investigations of slaughtered rabbits were thoroughly carried out for the gross lesions and any abnormal gross changes in the internal organs. Tissue specimens were taken and rapidly fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned (4–5µm) and mounted on glass slides. Sections were stained with hematoxylin and eosin (HE) for light microscopic examination **(Culling, 1983).**

4.3. Electron microscope trans-sectioning

Liver tissues were fixed immediately in 3% glutaraldehyde in 0.1 M Sodium cacodylate buffer (pH 7.3-7.4) for at least 4 hours at 4˚C and were sent to Zoology Department, College of Science, Ein shams University for electron microscopic examination. The specimens were washed overnight in the same buffer at 4 ºC and post-fixation in 2% osmium tetroxide in the same buffer for 2 h at 4°C. Specimens were rinsed twice in the same buffer for 10 min before dehydrated in ascending series of ethanol and finally were embedded in araldite. Semi-thin sections were cut with Leica ultracut UC7 and were stained with toluidine blue for light microscopic examination. Ultra-thin sections were stained with uranyl acetate and lead citrate and finally were examined with JEOL-JSM-1011 electron microscopy at 80–100 kV **(Al-Ghamdy et al., 2005; Ball et al., 2014).**

4.4. Molecular detection of *E. stiedae* in the liver tissue and blood

DNA was extracted from blood samples of infected rabbits slaughtered at (3,6 and 9) day post infection and from liver tissues at (3,6,9,12,15 and 18) day post infection using extraction kit (Biobasic , Inc., Canada, Cat. No. BS427) according to **Guven et al. (2013).** Following the kits instructions briefly; the samples were mixed with the kit lysis buffer, ACL solution (300 µL sample + 300 µL lysis buffer) and 20 µL Protienase K were added, and the mixture was incubated at 56˚C till lysis was complete (achieved after 3 hours). The DNA samples (100 µl) were collected after loading on the mini spin column of the kit. The DNA was eluted in 50 µL of elution buffer included in the same kit. DNA extracts were stored at -20°C for using in PCR.

DNA amplification:

Purified DNA extracts of infected liver or blood samples were analyzed with PCR. Primers (Es1F/R) according to Y**an et al. (2013)** (forward primer, ACCATGGGTCGGTTCGGTC, reverse primer ATGCGCGCGCCAACAAGCTAC). PCR master mix amplification kit (Gen aid) was used. PCR reactions were performed under the following conditions: initial denaturation for 3 min at 94˚C followed by 40 cycles of denaturation for 45 s at 94˚C, annealing for 45 s at 57˚C, and extension for 1 min at 72˚C. Final extension at 72˚C was allowed to proceed for 10 min. PCR products were detected in 1% agarose electrophoresis.

RESULTS

Parasitologically:

The first oocyst shedding in feces was observed on day 17 PI (prepatent period was 17 days), and oocysts were shed in considerable amounts until the end of the study. The peak period of oocysts shedding reached to the maximum in the 23, 24 and 25th days post infection then declined (Table 1).

Table 1: The oocysts count per gram of feces in last four sacrificed rabbits (3 gr. A and 1 gr. B)

Rabbit / Day	R1 (Rabbit. 1. infected)	R2 (Rabbit. 2 inf.)	R3 (Rabbit. 3 inf.)	Control uninfected Rabbit
17	500	100	140	0
18	1600	210	3600	0
19	3200	18000	13200	0
20	16000	42000	80000	0
21	33000	87000	250000	0
22	102000	195000	550000	0
23	131000	225000	600000	0
24	184000	240000	480000	0
25	900000	105000	500000	0
26	400000	120000	480000	0
27	360000	27000	200000	0

*****Shedding of oocyst begin from the 17 day post infection and the peak period reached to the maximum in the 23, 24 and 25th days post infection then declined.

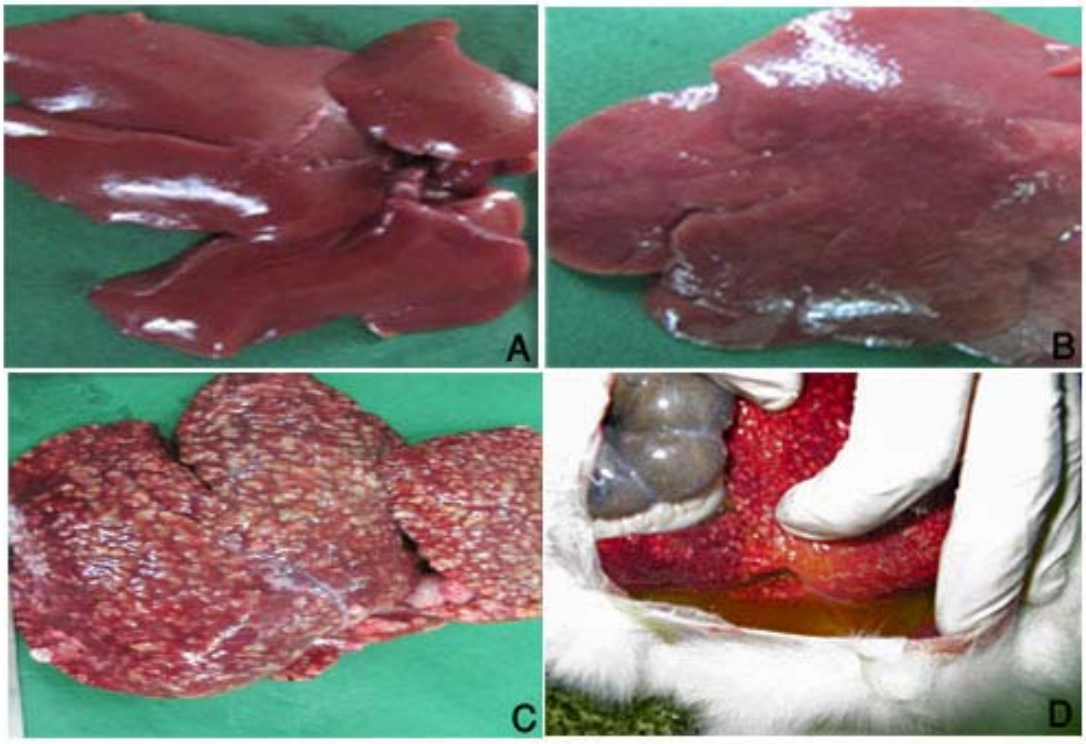

Figure 1: (A) Normal liver (B) liver at the 15th day post infection (C) liver at the27th day post infection (D) ascites at the 24th day post infection

Gross lesions:

The PM findings showed the liver had irregular yellowish white nodules scattered on the surface beginning from 15th day post-infection and became more prominent gradually, these nodules contained white caseous material. The parenchyma was severely congested and edematous. Hepatomegaly and ascites were obvious from the 21-24th days post infection. Gall bladder was distended with pale greenish fluid which turned yellowish at the end of study (Fig. 1).

Histopathological pictures:

The main histopathological appearance was the hyperplasia of the bile duct epithelial lining which begin from the 9th day post infection accompanied by degenerative changes and necrosis in the hepatic cells surrounded by inflammatory cells, then the hyperplasia of bile duct epithelium became more extensive forming long papillary projections and desquamation of some epithelial folds into the ductal lumen with the presence of mature schizonts and gametocytes in the epithelium of the bile

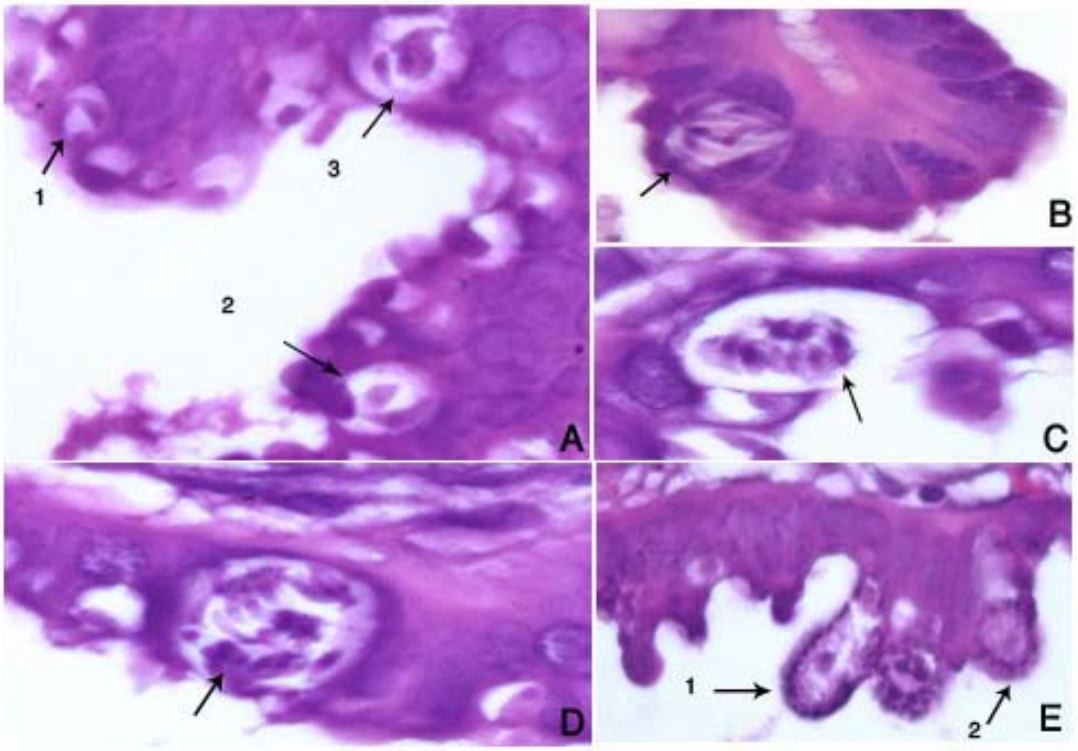

Figure 2: The different schizonts and gametocytes at the 15th day post infection: (A) 1-one merozoite 2-two merozoites 3- four merozoites (B) 8 merozoites (c) 16 merozoites (D) 32 merozoites (E) 1- macrogametocytes 2- microgametocytes.

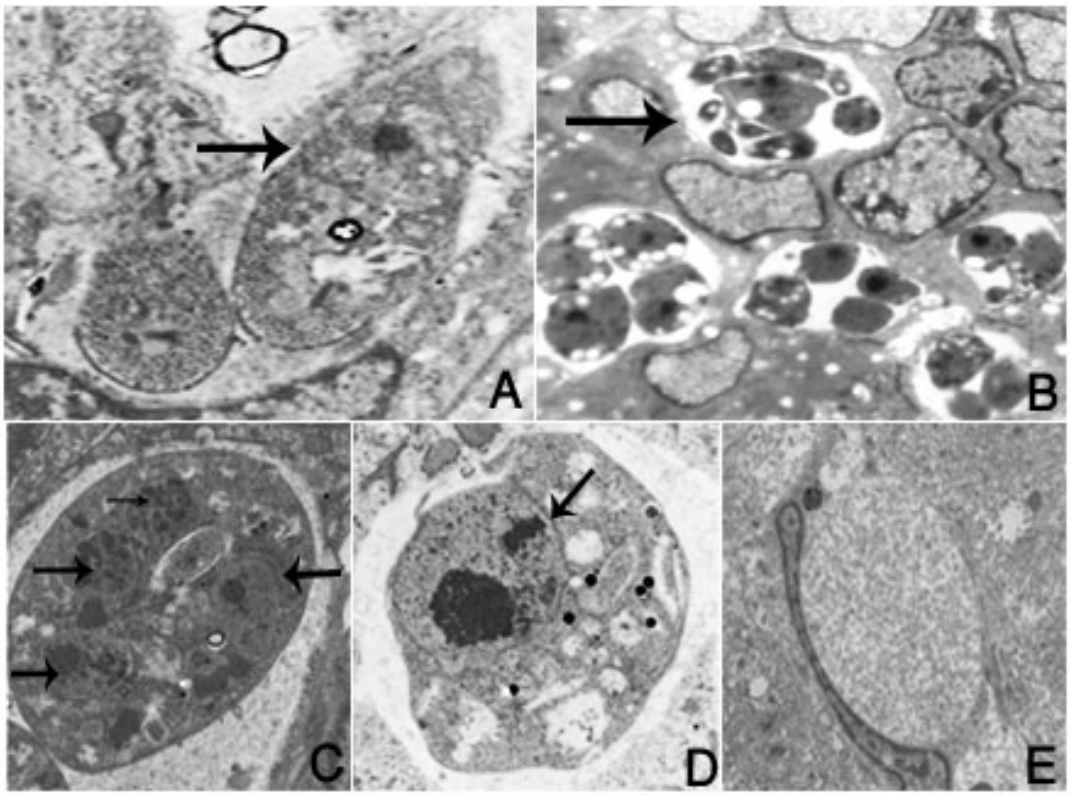

Figure 3: The developmental stages at the 15th day post infection and the oocysts in the bile duct epithelium at the 18th day post-infection. A- merozoite B-different schizonts C-microgamete (multinucleated) D-macrogamete (contain one nucleus) E-oocyst

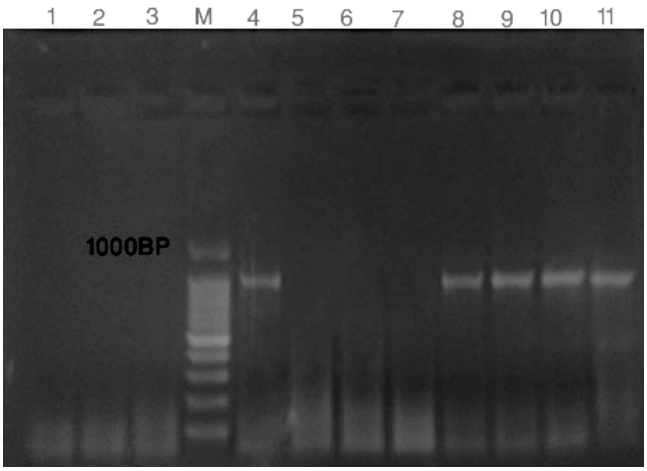

Figure 4: The blood samples were taken on days 3rd, 6th and 9th; are negative (lanes 1, 2 &3). The processed liver samples were taken on days 3rd, 6th and 9th; are negative (lanes 5,6,&7) and the samples of liver tissues that were processed for PCR from the 12th, 15th and 18th post infection were positive at the amplicon size 976 bp of *E. stiedae* with Agarose gel electrophoresis. In addition lane 4 control positive and lane 11 positive fecal sample.

ducts at the 15th day post-infection. Moreover, the hyperplastic bile ducts surrounded by large amounts of fibrous connective tissue infiltrated with mononuclear cells and the hepatic parenchyma showed dilation and congestion of central veins and sinusoids with the rupture of lining endothelial layer (Fig. 2).

Trans-sectioning by EM:

The results of electron microscope were parallel with that of histopathology, which revealed different shizonts and gametocytes in the epithelium of the bile ducts at the 15th day post infection and oocysts in bile duct epithelium at the 18th post-infection (Fig. 3).

PCR findings:

The collected blood samples from the zero day of infection then at the 3rd, 6th and 9th days post infection were negative for PCR. In addition, the PCR results showed that no evidence of sporozoites migration in liver tissues in the 1st week of infection till the 12th day post infection. Mean while, the samples of liver tissues that were processed for PCR from the 12th post infection and the next periods of investigation (15th, 18th tillthe end of the experiment) were found positive at the amplicon size 976bp of *E. stiedae* with Agarose gel electrophoresis (Fig. 4).

Discussion

In the present study, the macroscopic examination of liver of experimentally infected rabbits revealed the irregular yellowish white nodules scattered on the surface at the 15th day post-infection and became more prominent gradually, hepatomegaly and ascites were obvious from the 21-24th days post-infection. The selections are characteristic for *E. stiedae* infection **(Al-Naimi et al., 2012; Abed and Yakoob, 2013)**. Hepatomegaly was due to marked proliferation and distention of bile ducts forming nodules raised above the surface causing increase in the secretion of mucous appeared as a creamy white fluid in cut surface **(Al-Mathal, 2008; Al-Naimi et al., 2012)**. The occurrence of those coccidian nodules is due to the toxic effect of the protozoon settled in the liver **(Barriga and Arnoni, 1979)**.

Microscopically, the developmental stages of *E. stiedae* and most significant lesions were noticed in the biliary epithelium at the 15th day post infection. The histopathological observations are similar to those observed by **(El-Akabawy et al., 2004; Abu-Akkada et al, 2010; Al-Naimi et al., 2012)**. The proliferation of the bile duct epithelium might be due to the multiplication of *E. stiedae* within the epithelium lead to sinusoidal dilatation, associated with fibrosis in and around the cords lead to the obstructed hepatic blood flow especially in the portal veins by immensely proliferating and dilating bile ducts, the stagnation of the blood flow would also result in hepatocellular degeneration and atrophy of the cords **(Singla et al., 2000; Al-Naimi et al., 2012)**.

Examination by electron microscope revealed different schizonts in the epithelium of bile ducts at the 15th day post infection, this nearly in agreement with **Černá and Sénaud (1971)** and **Hung et al. (1984)** whom observed different schizogony stages at the 13th day post infection. Mean while **El-Masry (1983)** and **Li and Wang (1989)** mentioned that large number of fully mature schizonts containing various numbers of merozoites could be detected in most bile ducts at the 10th day post infection. Regarding the gametogony stages, were noticed in the epithelial of bile ducts at the 15th and 16th day post infection, this finding also reported by **Kheysin (1972)** and **El-Masry (1983)** whom recorded the gametogony stages at the (15 and 16th day post infection) respectively. Another studies by **Mining (1936)** and **Soulsby (1968)** found the gametogony stages in the 11-13th days post infection. The variance in schizonts and gamogony stages

may be referred to the rabbit breed, and the parasites strain in the view of authors.

The prepatent period was observed at the 17th day post infection, similar results were detected by **(Abu-Akkada et al, 2010; Abu-El-Ezz et al, 2012)**, slight difference recorded with the results of **Soulsby (1968)** and **Shameem and Devada (2005)**, whom found the prepatent period on the 18th day post infection. Comparable results were observed by **Levine (1973) and Cam et al. (2008)**, in which they recorded the first oocyst shedding on the 6-9th days post-infection. While **Jibike et al. (1995)** recorded prepatent period at the 11th days post infection. Moreover, **Gomez-Bautista et al. (1987)** and **Wang and Tsai (1993)** found the prepatent period at the (14-15th) day post infection respectively. In contrary, **Smetena (1933)** and **Barriga and Arnoni (1981)** reported that the first oocystic shedding appeared at the 3rd -4th week post-infection. The peak of oocyst shedding was recorded in the 23, 24 and 25thdays post infection then began to decline until the end of the study. These results were nearly parallel with **Kutkat et al. (1998)** and **Abdel-Megeed and Abu-El Ezz (2005)**, while **(Lammler and Hein, 1981; Abu-Akkada et al., 2010)** observed that the highest oocysts concentration was seen between the 17th-21st days post infection. On the other hand, **Balbaa et al. (2012)** recorded the maximum oocysts shedding at the 26th day post infection. This difference in the oocysts count might be due to *E. stiedae* oocysts infection dose and age susceptibility of rabbits **(Abu-El-Ezz et al., 2012)**. Also, we think that the animal breed may be of a role in this.

Purified DNA extracts of infected liver samples were analyzed with PCR. Primers (Es1 F/R) according to **(Yan et al., 2013)** who developed and used a specific and sensitive multiplex PCR diagnostic assay based on polymorphic sites of ITS1 and ITS2 to identify the three highly pathogenic species from rabbits, *E. stiedae*, *E. intestinalis*, and *E. flavescens.*

Consequently, this study investigated *E. stiedae* in liver tissue of experimentally infected rabbits with PCR. We succeeded to detect it from the 12th day post infection. In addition PCR results confirmed those obtained from parasitological, histopathological and electron microscopic examinations. Moreover, this early detection of infection helps in early treatment and before deleterious effects of *E. stiedae* are advanced.

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