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Original Article

Eimeria stiedae: Infection rate and molecular characterization by nested PCR in rabbits from Minoufiya Governorate, Egypt

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Abstract:

Eimeria stiedae infects the epithelium of bile ducts of the liver and causes economic losses for the rabbit industry. In this study, we aimed to study the infection rate and histopathology and to characterize *Eimeria stiedae* from Ashmoun and Sadat City, Minoufiya governorate, Egypt by a nested polymerase chain reaction. Specific PCR and nested PCR primers to the ITS-2 gene of *Eimeria stiedae* were used. The infection rate was 12.5%. The infection rate among localities was 19% for Ashmoun and 7% for Sadat. The infection rate among sex groups was 12.35% for females and 13.33% for males. The infection rate among age groups was 11.11% for >1year and 15.38% for <1year age groups. The infection rate was 8.75 for autumn, 18.33% for winter, 12% for spring, and 10% for summer. The locality had a significant effect on the infection rate (P< 0.0378), while the sex, age, and season did not significantly affect it. The histopathological lesions were identical to hepatic coccidiosis caused by *E. stiedae* and its stages were observed. There was hydropic degeneration in hepatocytes. The infection significantly increased ALT, AST, ALP, GGT, total bilirubin, direct bilirubin, indirect bilirubin, and urea while decreased PCV, Hb, RBCS, MCH, MCHC, and lymphocytes. The PCR and nested PCR amplified the expected bands of the ITS-2 gene. The PCR and nested PCR products were sequenced. The sequences had high identity percent with *E. stiedae* isolates from China (LY) and Egypt (BSU-1). Nested PCR of the ITS-2 gene might be useful in the molecular diagnosis of *E. stiedae* in rabbits.

Keywords: *Eimeria stiedae*; Infection rate; Nested PCR; ITS-2; Hematology; Biochemical parameters; Histopathology; Minoufiya; Egypt

INTRODUCTION

The Phoenicians first encountered the rabbit in Spain around 1000 B.C. Rabbit farming has tremendous potential in developing countries improving food safety and quality. Because of the short pregnancy and pronounced prolificacy, the rabbits are extremely productive. A female rabbit can produce about 80 kilos (kg) of meat annually, i.e. 2900-3000% of its weight of the meat. Rabbit meat is highly nutritious, juicy, cholesterol-free, and high in calcium, vitamins, and minerals. Rabbits rising from a backyard give smallholder farmers additional income and increase the food of provincial and urban individuals **(FAO, 2001)**. For household output, rabbits suit well high labor

and investment costs. Rabbits are herbivores and do not fight for food with humans. World rabbit production amounts to over 1 million tons per year from which Egypt delivered 69,600 tonnes **(FAO, 2001)**. Animal diseases are of the general fields of restrictions on widespread rabbit farming **(FAO, 2001)**. Protozoan parasites are from these restrictions.

Eimeria stiedae is an important protozoan of rabbits. It infects the liver and reproduces in the epithelial lining of the bile ducts **(Bangoura and Daugschies, 2018)**. It causes high profitable losses for rabbit production. Clinical signs include anorexia, depression, brown watery diarrhea, emaciation, harsh hair coating, droopy and swollen abdomen with progressive weakness, jaundice, and death **(Pakandl, 2013)**. Clinical signs only appear on the young rabbits and may lead to their death while the adults are usually carriers **(Okerman, 1988)**. The lesions typically bound to liver and swollen bile ducts **(Al-Naimi et al., 2012)**. Macroscopically, the liver is enlarged and has numerous small white nodules on its surface **(Wang and Tsai, 1991)**. The histopathological changes comprise hyperplasia and hypertrophy of the bile duct epithelium with the developing stages of *E. stiedae* **(Darzi et al., 2007)**.

Eimeria stiedae infection was recorded in rabbits from different geographical areas either clinically or microscopically using parasitological or histopathological techniques such as in Taiwan **(Wang and Tsai, 1991)**, Iraq **(Al-Naimi et al., 2012)**, Kingdom Saudi Arabia **(Abdel-Baki and Al-Quraishy, 2013; Al-Mathal, 2008; Toula and Ramadan, 1998)**, Iran **(Tehrani et al., 2013)**, India **(Darzi et al., 2007; Singla et al., 2000; Sivajothi et al., 2016)**, Australia **(Stodart, 1971)**, Portugal **(Silva et al., 2015)**, Poland **(Nosal et al., 2006)**, Brazil (de Almeida et al., 2006), Kenya **(Okumu et al., 2014)**, and Egypt **(El-Shahawi et al., 2012)**. Complement fixation test was applied for diagnosis in a former study **(Rose, 1961)**. Recently, molecular tools were utilized for its identification in some studies **(Faraj, 2017; Hassan et al., 2016; Ütük et al., 2015; Yan et al., 2013)**. In this research, we assessed the utilization of a nested PCR targeting the ITS-2 gene for characterization of *Eimeria stiedae* in rabbits from Minoufiya, Egypt.

MATERIALS AND METHODS

1. Animals

Two hundred rabbits were collected, 100 from Sadat City and 100 from Ashmoun. The age of collected rabbits was arranged into two groups135 rabbits were more than one year and 65 were less than one year. The sex groups included 170 females and 30 males. Animals were examined in the period from April 2019-Aprile 2020.

2. Fecal analysis

Feces from all collected animals were examined by the flotation technique **(Foreyt, 1989)**.

3. Parasite

Eimeria stiedae was collected from infected rabbits from Ashmoun and Sadat City districts, Minoufiya governorate, Egypt. Oocysts were collected from the fecal samples from Sadat City as delineated by **Foreyt (1989)**. Oocysts collected from liver samples from Ashmoun by macerating the liver and filtration to detain out the coarser particles of the minced tissues by passing the mixture through several sieves followed by centrifugation **(Rose, 1961)**. Oocysts were sporulated using potassium dichromate 2.5%

(MAFF, 1977).

4. Histopathology

Five liver tissue samples with lesions were collected from positive animals in 10% neutral buffered formalin (NBF) for histopathological examination. Thin sections were prepared from liver tissues and stained with H and E **(Bancroft et al., 1996)**.

5. Blood samples:

Twenty Blood samples were collected from the rabbits either infected or non-infected with *E. stiedae* in tubes enclosing EDTA for measuring hematological parameters. Other blood samples were collected in plain centrifuge tubes and serum samples were separated and stored at -20°C until evaluated for the selected biochemical parameters.

6.Hematological and biochemical analysis

Hematological variables included packed cell volume (PCV), hemoglobin concentration (Hb), red blood cell counts (RBCs), Mean corpuscular volume (MCV), Mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), total leukocyte count (TLC) and differential leukocyte count were performed according to the routine blood procedures adopted by Feldman et al. (2000). Activities of Serum enzymes included alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and Gamma-glutamyl transferase (GGT) and concentrations of total protein (TP), albumin (Alb), blood urea (U), creatinine (Cr), total bilirubin (TB), direct bilirubin (DB), indirect bilirubin (IB) were measured spectrophotometrically using Spinreact diagnostic kits (Spain) and following the manufacturer's instructions.

7. DNA extraction

Oocysts were broken by sonication **(Hassan et al., 2016)**. DNA was extracted using G-spin^{TM} Total DNA extraction kit (iNtRON, Seoul, Korea). DNA was quantified using NanoDrop 2000c (Thermo Scientific, USA).

8. Polymerase chain reaction (PCR), and Nested Polymerase chain reaction (nPCR)

The PCR and nested PCR were performed consistent with previous studies **(AbouLaila et al., 2010a,b)** with some modifications. A 25 µL reaction volume contains Dream Taq^{TM} Green PCR Master Mix (2X) (Thermo Fisher Scientific Inc., California, USA), 120 ng of DNA, 50 µM of each primer, and nuclease-free water. The PCR reaction was completed utilizing forward 5$^{'}$ - GCAACGGCGTGCAGGGTC TA-3$^{'}$ and reverse 5$^{'}$ - CACTACTACTCTACCTTCCGC-3$^{'}$ primers (Table 2) for ITS-2 gene of *Eimeria stiedae*, which were deliberate dependent on ITS-2 gene sequences in 18S rRNA, ITS-1, 5.8S rRNA, ITS-2, and 28S rRNA region gene bank accession numbers JQ328190 and KU886239 and targeting a sequence of 393 bp from 692 to 1084 and from 504 to 896 bp, respectively. The SimpliAmp^{TM} Thermal Cycler (Applied Biosystems, Singapore) was used to conduct the PCR reaction. The PCR conditions comprised: denaturation at 95 ° C for 3 minutes and 35 cycles of denaturation at 95 ° C for 30 seconds, annealing at 58 ° C for 30

seconds, extension at 72 ° C for 30 seconds, and a concluding extension at 72 ° C for 7 minutes. Half microliter of the PCR product was utilized in the nested PCR reaction with the nested ITS-2 forward f1 5' - GTGCTGTTGCTGATCTCCTT-3' and reverse r1 5' - CGTACACATGCAACAAC CTC 3' primers (Table 2), which produce an amplicon size of 169 bp. The nested fragment is located at positions 821-989 and 633-801 bp of ITS2 gene in *E. stiedae* 18SrRNA, ITS-1, 5.8SrRNA, ITS-2, and 28SrRNA region gene bank accession numbers JQ328190 and KU886239, respectively. The nested PCR reaction conditions were equivalent to PCR. Five µL of the PCR and nested PCR products were exposed to electrophoresis with a DNA ladder size marker on 2 % agarose gel stained with ethidium bromide.

9. Sequence analysis

For DNA sequencing, a 50 µL reaction volume was prepared for each of the PCR and nested PCR reactions. The DNA was cleaned using Wizard® SV Gel and PCR Clean-Up Systems (Promega, Madison, USA) and submitted for sequencing (Macrogen, Seoul, Korea). The nucleotide sequences of the PCR and nested PCR were searched by BLAST® (Basic Local Alignment Search Tool) at the NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Then, they were referred to the gene bank to get accession numbers. The neighbor-Joining phylogenetic tree of the *E. stiedae* ITS-2 was made using CLUTAL X 1.8 and NJplot 2.4. The sequences that were used in the tree construction were *E. stiedae* Ashmoun (LC491616), *E. stiedae* Sadat (LC491618), *E. stiedae* Bein-Suef strain BSU-1/Egypt (KU886239), *E. stiedae* strain LY/China (JQ328190), *E. intestinalis* (JX406874), *E. flavescens* (JX406873), *E. irresidua* (JX406875), *E. magna* (JX406876), *E. media* (JX406877), *E. ontarioensis* (EU302686), *E. papillata* (AY779499), and *Arthroderma cuniculi* (AJ390382) as out-group. Identity percent with other *Eimeria* species infecting rabbits and mice were detected using MAFTT online software at EBI.

10. Statistical analysis

A Chi-Square test was used to analyze differences between age, sex, season, and locality groups. All values for hematological and biochemical analyses were presented as mean ± standard error (SE). The mean laboratory values of infected and non-infected groups were compared by Student's *t*-test. SPSS 19 (IBM Corp., Armonk, New York, USA) was used to perform the analysis. 0.05 level of probability is accepted as significant.

RESULTS

Infection rate

The infection rate was 12.5% (25/200) (Table 1). The infection rate among localities was 19% (18/100) for Ashmoun and 7% (7/100) for Sadat City (Table 1). The infection rate among sex groups was 12.35% (21/170) for females and 13.33% (4/30) for males (Table 1). The infection rate among age groups was 11.11% (15/135) for > 1-year and 15.38% (10/65) for < 1-year age groups (Table 1). The infection rate was 8.75 (7/80) in autumn, 18.33% (11/60) in winter, 12% (6/50) in spring, and 10% (1/10) in summer (Table 1). The locality had a significant effect on the infection rate (X^2 = 4.313 and P< 0.0378), while the sex, age, and season did not significantly affect it (Table 1).

Histopathology

Macroscopical lesions

The liver was enlarged in the infected

animals. The liver surface was occupied with yellowish to white nodules of different sizes.

Microscopical lesions

Microscopically, the developing stages of the *E. stiedae* were distinguished in the multiplied epithelial-finger like projections in the lumen of the bile duct. The affected bile ducts were surrounded by a thick layer of fibrous connective tissue and the adjacent hepatocytes showing hydropic degeneration (Fig.1 A & B). The proliferated biliary epithelium comprises the developing schizonts, microgametocytes, macrogametocytes, and the shelled oocysts (Fig. 1 C). The hepatocytes surrounding the affected bile ducts showing hydropic degeneration in their cytoplasm (Fig. 1 D).

Hematology

Data in the table (3) implicated that rabbits infected with *E. stiedae* showed hematological changes such as a significant decrease in the values of PCV, Hb, RBCS, MCH, MCHC, and lymphocytes. While the values of TLC and percentages of neutrophils and eosinophils increased significantly. MCV and monocytes' values showed no significant differences.

Biochemical analysis

Table 4 revealed that rabbits infected with *E. stiedae* had a significant increase in the values of ALT, AST, ALP, GGT, total bilirubin, direct bilirubin, indirect bilirubin, and urea, whereas the values of total protein and albumin decreased significantly. The ratios of albumin to globulin and the values of creatinine showed no significant difference.

Nested Polymerase chain reaction (nPCR) and Sequence analysis

The PCR reaction utilizing primers F and R for ITS-2 of *E. stiedae* resulted in the amplification of the expected band size of 394 bp (Fig.2, lanes 1, 2, and 3) while no amplification from the double-distilled water control (Fig.2, lane 4). The PCR primers amplified DNA extracted from the liver (Fig.2, lane1) and fecal (Fig.2, lanes 2 and 3) samples from Ashmoun and Sadat City districts, respectively. The nested PCR primers f1 and r1 amplified the estimated band size of 169 bp (Fig.3, lanes 2, 3, and 4). No amplification was detected from negative DDW control (Fig.3, lane 1). The nPCR primers amplified DNA extracted from the liver (Fig.3, lane 2) and fecal (Fig.3, lanes 3 and 4) samples from Ashmoun and Sadat City districts, respectively. Nucleotide blast search (blastn) using BLAST® at NCBI showed that the sequence of PCR products from Ashmoun district (accession no.: LC491616) had a percent identity of 96.18 % and 94.68 % with *E. stiedae* China/LY and Egypt/BSU-1 isolates accession numbers JQ328190.1 and KU886239.1, respectively. The sequence of PCR products from Sadat City district (accession no.: LC491618) had a percent identity of 96.12 % and 94.01 % with *E. stiedae* China/LY and Egypt/BSU-1 isolates accession numbers JQ328190.1 and KU886239.1, respectively. The sequence of nPCR products from Ashmoun district (accession no.: LC491617) had a percent identity of 98.57 % and 97.86 % with *E. stiedae* China/LY and Egypt/BSU-1 isolates accession numbers JQ328190.1 and KU886239.1, respectively. The sequence of nPCR products from Sadat City district (accession no.: LC491619) had a percent identity of 96.12 % and 94.01 % with *E. stiedae* China/LY and Egypt/BSU-1 isolates accession numbers JQ328190.1 and

KU886239.1, respectively. The MAFFT program showed that *E. stiedae* Ashmoun (LC491616) had identity percent of 63.77%, 60.11%, 58.56%, 52.68%, 47.8%, 50%, and 56.18% with *E. intestinalis* (JX406874), *E. magna* (JX406876), *E. media* (JX406877), *E. flavescens* (JX406873), *E. irresidua* (JX406875), *E. ontarioensis* (EU302686), and *E. papillata* (AY779499), respectively. *E. stiedae* Sadat (LC491618) had identity percent of 71.03%, 63.39%, 59.82%, 56.12%, 48.3%, 52.4%, and 56.12% for *E. intestinalis* (JX406874), *E. magna* (JX406876), *E. media* (JX406877), *E. flavescens* (JX406873), *E. irresidua* (JX406875), *E. ontarioensis* (EU302686), and *E. papillata* (AY779499), respectively. *E. stiedae* from Ashmoun and Sadat City occurred in the same clade with other *E. stiedae* from Egypt and China (Fig.4).

DISCUSSION

In the present study, the infection rate was 12.5% and had a similar range,11.5%, with that reported in Kenya **(Okumu et al., 2014)** while higher than those of 5% and 3.34% reported in Saudi Arabia **(Abdel-Baki and Al-Quraishy, 2013)** and Poland **(Szkucik et al., 2014)**, respectively. The infection rate is lower than those of 57.28% reported in India **(Galal, 2007)**, 32.24% in Saudi Arabia **(Al-Mathal, 2008)**, 27% in Finland **(Mäkitaipale et al., 2017)**, 26.87% in Iran **(Tehrani et al., 2013)**, and 17.5% in Iraq **(Khider et al., 2015).** Differences in prevalence may be due to variations in environmental conditions, and breeds of rabbits. The infection rate among localities was 19% for Ashmoun and 7% for Sadat City this difference may be due to the rural nature of Ashmoun where many farmers reared rabbits than the urban nature of Sadat City. The infection rate among sex groups was a similar range and lower than 33.46 and 31.02% in Saudi Arabia **(Al-Mathal, 2008),** 20% and 16% in Iraq **(Khider et al., 2015)**, but higher than 4.7% and 5.3% in Iran **(Tehrani et al., 2013)** for male and female rabbits, respectively. The infection rate was higher in young age < 1-year than > 1-year groups. This might be because young rabbits are less immune to infection and this agreed with results reported by **Mäkitaipale et al. (2017)** and **Tehrani et al. (2013)** while this disagreed with **Al-Mathal (2008)** who detected higher infections in older rabbits in farms. This may be due to differences in rearing systems used for raising rabbits and low sanitary conditions in these farms **(Al-Mathal, 2008)**. The infection rate was the highest in winter followed by spring while it was the lowest in autumn.

In this study, we observed that the hepatocytes around the affected bile ducts showed severe hydropic degenerative changes. According to our knowledge, the previously published data did not discuss the relationship between the presence of *E. stiedae* in rabbit bile ducts and the cellular hydropic degeneration of hepatocytes. Additionally, it was reported that *Schistosoma mansoni* induces hydropic degeneration in hepatocytes of the infected mice **(Al Hamshary et al., 2018)**, severe hydropic degeneration in hepatocytes with vacuolated cytoplasm and vesicular nucleus **(Abdelgelil et al., 2019)**. The association concerning the existence of *E. stiedae* in rabbit bile ducts and the cellular hydropic degenerative changes in hepatocytes may be due to the toxic metabolites of the parasites but this point needs further investigation. Furthermore, histopathological changes were similar to the ones in prior work for both post mortem lesions of hepatic coccidiosis and microscopy **(Al-Naimi et al., 2012; Darzi et al., 2007; Okumu et al., 2014; Sanyal and Sharma, 1990; Singla et al., 2000;**

Table (1): Effect of sex, age, locality, and season on *Eimeria stiedae* infection in rabbits from Minoufiya Governorate, Egypt

		Number examined	Number infected	Percent	X^2	*P*-value*
Sex	Male	30	4	13.33	0.01732	0.8953
	Female	170	21	12.35		
Age	> 1year	135	15	11.11	0.5625	0.4533
	<1 year	65	10	15.38		
Locality	Ashmoun	100	18	18	4.313	0.0378*
	Sadat City	100	7	7		
Season	Spring	50	6	12	2.262	0.5198
	Summer	10	1	10		
	Autumn	80	7	8.75		
	Winter	60	11	18.33		

*Significant at $P < 0.05$

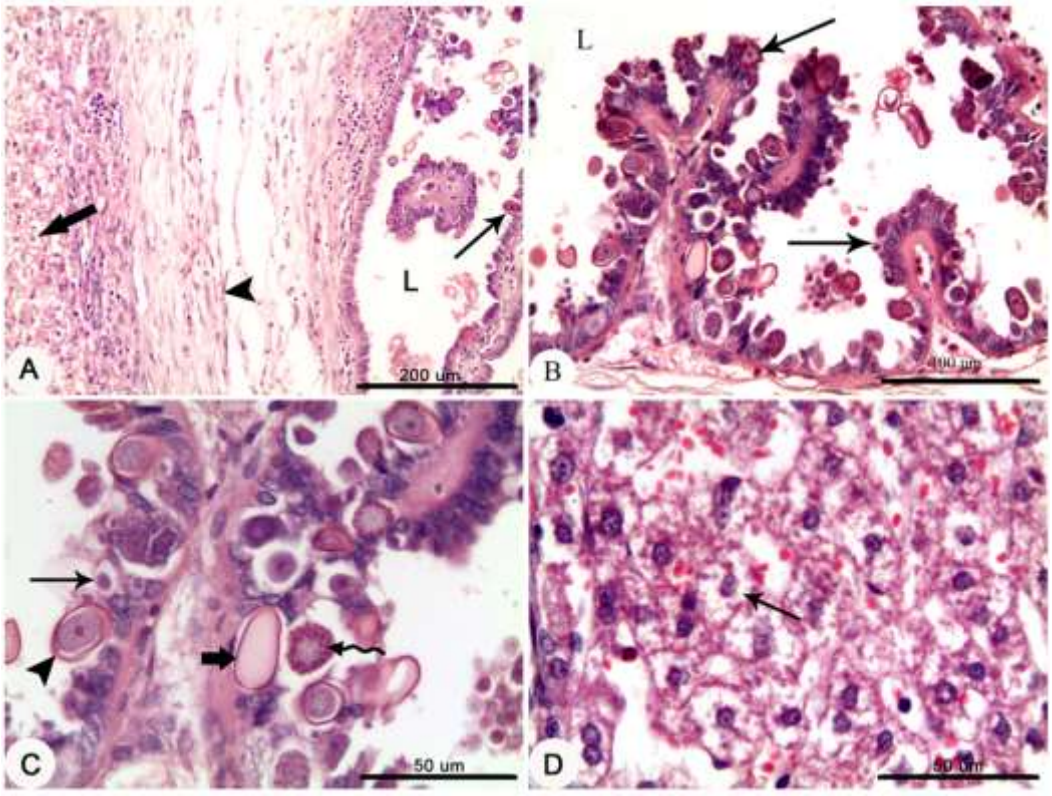

Fig. (1): *Eimeria stiedae* infection in rabbit liver, H & E stain. **A)** Showing developmental stages of the parasite (thin arrow) in the lumen of the bile ducts (L) which surrounded by a thick layer of fibrous connective tissue (arrowhead) and the adjacent hepatocytes showing hydropic degeneration (thick arrow), Bar 200 um. **B)** Showing finger-like projections having the developmental stages of the parasite (arrow) in the lumen of the bile ducts (L), Bar 200 µm. **C)** Showing the biliary epithelium contains developing schizonts (thin arrow), a zygote (arrowhead), macrogametocytes (bent arrow), and the shelled oocysts (thick arrow), Bar 50 µm. **D)** Showing hydropic degeneration in hepatocytes (arrow), Bar 50 µm.

Table (2): Sequences of *Eimeria Stiedae* ITS-2 PCR and nested PCR primers

Reaction	Primer	Sequence (5´→3´)	Amplicon Size
PCR	F	GCAACGGCGTGCAGGGTCTA	393 bp
	R	CACTACTACTCTACCTTCCGC	
Nested PCR	f1	GTGCTGTTGCTGATCTCCTT	169 bp
	r1	CGTACACATGCAACAACCTC	

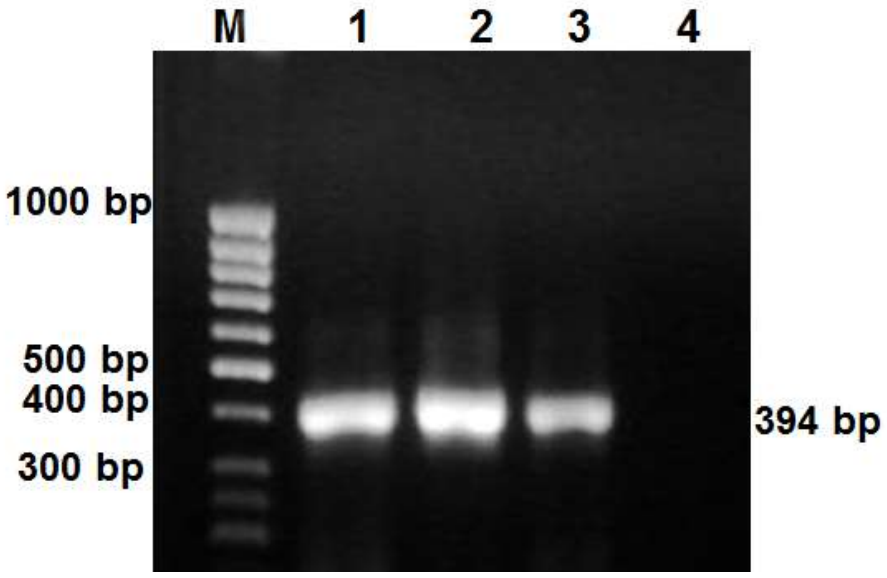

Fig. (2): Polymerase chain reaction (PCR) amplifies the ITS2 gene of *E. stiedae* using F and R primers. Lane 1, a liver sample from Ashmoun district, lane 2 and 3, the fecal samples from Sadat City district, and lane 4, negative control double-distilled water. M is a DNA molecular size marker.

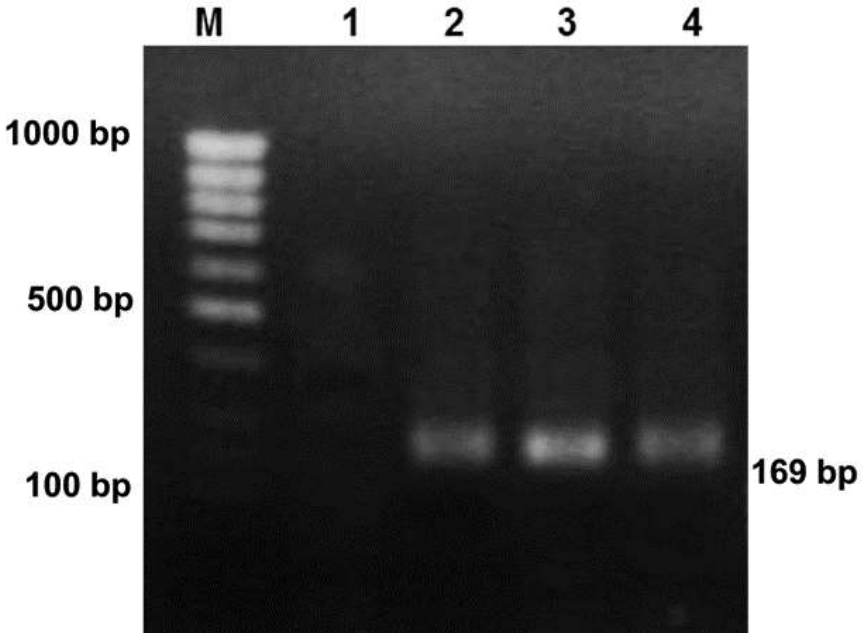

Fig. (3): Nested polymerase chain reaction for ITS-2 gene from *E. stiedae* using primers f1 and r1. Lane 1, negative control double-distilled water, lane 2, liver sample from Ashmoun, and lanes 3 and 4, fecal samples from Sadat City, M, DNA molecular size marker.

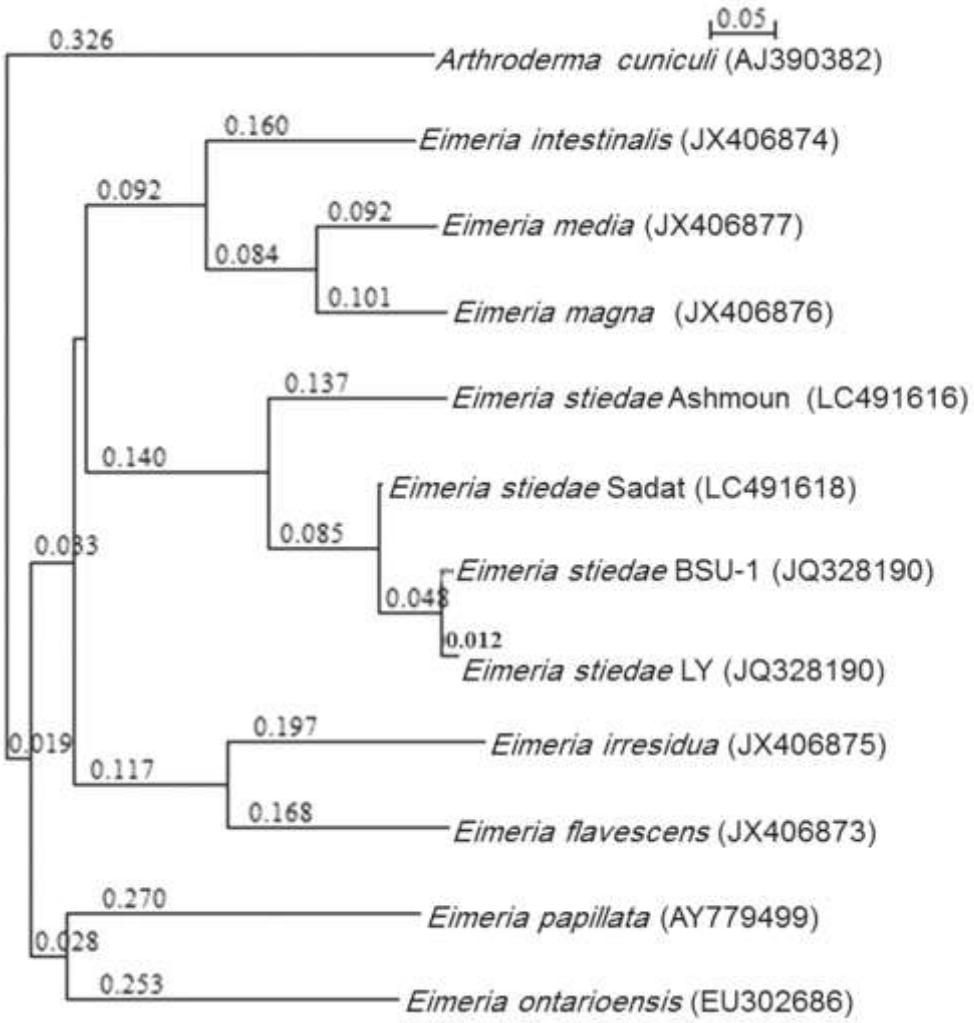

Fig. (4): Neighbor-joining Phylogenetic tree of the *Eimeria stiedae* ITS-2. The sequences that were used in the tree construction were *E. stiedae* Ashmoun (LC491616), *E. stiedae* Sadat (LC491618), *E. stiedae* BSU-1/Egypt (KU886239), *E. stiedae* LY/China (JQ328190), *Eimeria intestinalis* (JX406874), *Eimeria flavescens* (JX406873), *Eimeria irresidua* (JX406875), *Eimeria magna* (JX406876), *Eimeria media* (JX406877), *Eimeria ontarioensis* (EU302686), *Eimeria papillata* (AY779499), and *Arthroderma cuniculi* (AJ390382) as an outgroup. The tree showed the scale bar.

Table (3): Hematological variables in rabbits infected with *E. stiedae* compared to non-infected control

Parameters	Groups (No= 20)*	
	Control	Infected
PCV (%)	39.65±0.41 [a]	36.70±0.36 [b]
Hb (g/dl)	12.70± 0.22 [a]	10.07±0.07 [b]
RBCs (x10$^{6)}$	6.23±0.09 [a]	5.82±0.08 [b]
MCV (fl)	63.85±0.79 [a]	63.26±0.93 [a]
MCH (pg)	20.49±0.46 [a]	17.39±0.31 [b]
MCHC (%)	32.01±0.34 [a]	27.53±0.47 [b]
TLC (x10^3)	7.44±0.18 [b]	10.28±0.11 [a]
Neutrophils (%)	31.00±0.36 [b]	41.25±0.19 [a]
Lymphocytes (%)	64.00±0.32 [a]	52.50±0.41 [b]
Monocytes(%)	4.60±0.21 [a]	4.60±0.22 [a]
Esinophils (%)	0.40±0.11 [b]	1.65±0.15 [a]

*Values are means ± SE. Means in the row without a common letter differ significantly at ($P<0.05$).

Table (4): Blood biomarkers in rabbits infected with *E. stiedae* compared to the non-infected control

Parameters	Groups (No= 20)*	
	Control	Infected
ALT (U/L)	20.09±0.45 [b]	25.17±0.35 [a]
AST (U/L)	11.00±0.23 [b]	17.00±0.36 [a]
ALP (U/L)	198.32±3.91 [b]	267.50±2.36 [a]
GGT (U/L)	26.77±0.44 [b]	37.96±0.77 [a]
TP (g/dl)	7.49±0.11 [a]	6.84±0.19 [b]
Albumin (g/dl)	3.79±0.01 [a]	3.46±0.06 [b]
Globulin (g/dl)	3.71±0.12 [a]	3.39±0.15 [a]
AG	1.04±0.04 a	1.05±0.03 [a]
T Bilirubin (mg/dl)	1.17±0.00 [b]	1.58±0.07 [a]
D Bilirubin (mg/dl)	0.24±0.00 [b]	0.42±0.04 [a]
I Bilirubin (mg/dl)	0.93±0.01 [b]	1.16±0.04 [a]
Urea (mg/dl)	42.89±0.37 [b]	44.75±0.45 [a]
Creatinine (mg/dl)	1.16±0.00 [a]	1.16±0.00 [a]
ALT (U/L)	20.09±0.45 [b]	25.17±0.35 [a]

*Values are means ± SE. Means in the row without a common letter differ significantly at ($P<0.05$).

Tehrani et al., 2013).

The results indicated anemia that was evidenced by low PCV, Hb, RBCs, MCH, and MCHC values. However, MCV values were insignificant. **Freitas et al. (2011)** reported anemia in rabbits infected with *E. stiedae* due to decreased values of RBCs, Hb, and hematocrit (Hct). Moreover, **Hana et al. (2011)**, observed anemia after *E. magna* infection in rabbits. This study supports what was assumed with **Harvey (2008)** and **Petrova et al. (2018)** who stated that lower values of PCV, Hb, and RBCs were due to liver damage and subsequent inflammation (anemia of inflammatory diseases). Also, the pathophysiology of anemia of inflammatory disease (AID) includes shortening the life span of red cells, inhibition of iron metabolism, and impaired erythropoietin-mediated erythropoiesis in the bone marrow **(Fry, 2010)**.

Belonging to leukogram, there was a significant increase in total leukocyte values and percentages of neutrophils and eosinophils with a significant reduction in calculations of lymphocytes. Leucocytes are components of the defense mechanisms opposing foreign agents such as parasites. *E. stiedae* induces an inflammatory reaction in the infected rabbits, which leads to the transfer of leukocytes to the areas of inflammation **(Cam et al. 2008; Freitas et al. 2011; Petrova et al., 2018)**. Neutrophilia is produced by two mechanisms during infection. In the inflammatory mechanism, cytokine stimulation to bone marrow induces releasing the storage pool of post-mitotic mature and immature band neutrophils, which brings about a neutrophilia with a left-shift **(Weiss and Wardrop, 2010)**. Moreover, the second mechanism depends on the stressful process caused by the disease that increases cortisol levels which have a direct impact on the production and distribution of white cells and lead to a relative neutrophilia and lymphopenia **(Toth and Krueger, 1989)**. The increase in the percentages of neutrophils caused the total white blood cell count to increase. Eosinophils are part of the granulocytes and their increasing serves as an indicator of the parasitic diseases. Eosinophilia has been observed in rabbits experimentally convinced chronic ascarid infection **(Gupta and Trivedi, 1981)**.

Regarding changes in biochemical parameters, *E. stiedae* caused an increase in liver enzyme activities (ALT, AST, ALP, and GGT) further to an increase in total, direct, and indirect bilirubin. In rabbits, liver ALT activity is lower than other species **(Rosenthal, 1997)**, AST is established in liver, heart, skeletal muscle, kidney, and pancreas, with the maximum activity in the liver and skeletal muscle **(Benson and Paul-Murphy, 1999)**, Liver GGT is present primarily in the epithelial cells of the bile duct and therefore it is a signal of hepatobiliary disease rather than hepatocellular damage **(McLaughlin and Fish, 1994)**. Alkaline phosphatase is found in almost all tissues and is found united with cell membranes and particularly in the intestinal epithelium, renal tubules, osteoblasts, liver, and placenta **(McLaughlin and Fish, 1994)**. The main target for *E. stiedae* is the liver and bile ducts. **Manjunatha et al. (2019)** reported that *E. stiedae* caused hepatic coccidiosis evident by elevated ALT, AST, ALP, and total bilirubin. Furthermore, Cam et al. (2008) attributed the increases in AST, ALT, and GGT serum activities to hepatocellular damage and cholestasis. Hepatocellular injury releases ALT and AST from damaged hepatocytes and increases their activities in the serum **(Kim et al., 2008)**. However, high activity of ALP **(Fyffe and Wilson, 2010)** and high levels of total bilirubin **(Barriga and Arnoni;**

1981) indicate obstructive jaundice. The abundance of bile duct epithelium as well as many oocysts in the lumen of the bile duct resulting in obstructive jaundice **(Sanyal and Sharma, 1990)**. The liver is the only place for the synthesis of albumin and hypoalbuminemia is a character of progressive liver disease. In rabbits, *E. stiedae* causes hepatic coccidiosis. Severe infestations can lead to decreased albumin levels. Non-hepatic reasons for low serum albumin comprise glomerulopathy, protein-losing entero-pathy, malabsorption, and cardiac dropsy **(Varga, 2014)**. Hypoalbuminemia could be the reason of hypoproteinemia. Urea is a nitrogenous waste product formed in the liver as the outcome of the deamination of amino acids. It is transferred in the blood to the kidneys where it is discharged in the urine. Increased values of blood urea have been reported in rabbits being experimentally induced with coccidiosis **(Licois et al., 1978)** and this rise in urea levels has been explained as a result of intense nitrogen catabolism during disease-related weight loss, while **Yaplito-Lee et al. (2013)** attributed the elevation in urea levels to a disturbance in the urea cycle in the liver.

The PCR and nested PCR primers for the ITS-2 gene of *E. stiedae* successfully amplified the expected band size of 394 bp and 169 bp, respectively, from DNA extracted from oocysts collected from the liver and fecal samples. The sequences of the ITS2 gene from Minoufiya were highly identical to the sequences of ITS-2 of *E. stiedae* from China/LY and Egypt/BUS-1 isolates and occurred in the same phylogenetic clade; therefore, confirmed the infection with *E. stiedae* and indicate that nPCR might be used for molecular characterization of *E. stiedae.*

In conclusion, this study presented the infection rate of *E. stiedae* in rabbits from Minoufiya Governorate, Egypt and showed that ITS-2 PCR and nPCR successfully amplified *E. stiedae* DNA and might be used for its molecular characterization.

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الملخص العربي

إيميريا ستيدي: معدل الإصابه و التوصيف الجزيئي بالنيستد بي سي أر في محافظة المنوفيه بمصر

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تهاجم الإيميريا ستيدي الخلايا المبطنه للأوعيه المراريه في الكبد و تتسبب في خسائر إقتصاديه لصناعة الأرانب. نهدف في هذا البحث لدراسة معدل العدوي و التأثير الهيستوباثولوجي للايميريا ستيدي و التوصيف الجزيئي لها بإستخدام نيستد بي سي أر من مركزي اشمون ومدينة السادات بمحافظة المنوفيه بمصر. تم إستخدام باديئات للبي سي ار و النيستد بي سي ار خاصه بجين اي تي اس-٢ (ITS-2) من الإيميريا ستيدي. كان معدل الإصابه العام بين الأرانب ١٢.٥% و كان معدل الإصابه ١٩% من أشمون و ٧% من مدينة السادات. كان معدل الإصابه بين الأجناس المختلفه ١٢.٣٥ % للإناث و١٣.٣٣% للذكور. سجلت الأعمار الصغيره أقل من سنه معدل إصابه ١٥.٣٨% بينما سجلت الأعمار الكبيره أكبر من سنه معدل ١١.١١%. كانت معدلات الإصابه في الفصول المختلفه ٨.٧٥% و ١٨.٣٣% و ١٢%و ١٠% لكل من الخريف و الشتاء و الربيع و الصيف على الترتيب. كان هناك تأثير معنوي للعامل المكاني على نسبة الإصابه بينما لم يكن هناك تأثير معنوي للعمر او الجنس او فصول السنه. أكد الفحص الهيستوباثولوجي إصابة الكبد بالإيميريا ستيدي وكانت الأعراض التشريحيه و الميكروسكوبيه مطابقه للكوكسيديا الكبديه و شوهدت المراحل المختلفه للطفيل داخل خلايا الأوعيه المراريه و لوحظ وجود تنكس مائي في سيتوبلازم الخلايا الكبديه المجاوره للوعاء المراري المصاب. تسببت الإصابه بالايميريا ستيدي بزياده معنويه في إنزيمات الكبد و اليوريا و الكرياتينين و إنخفاض في خلايا الدم الحمراء و الخلايا الليمفاويه والهيموجلوبين. أنتج البي سي ار و النيستد بي سي أرللاي تي اس-٢ جين (ITS-2) الباندات الخاصه بالايميريا ستيدي بالحجم المتوقع. تم تحليل تتابع النيوكليتيدات و التركيب الجيني لنواتج البي سي ار و النيستد بي سي ار. ووجد ان تتابع النيوكيتيدات لهذه المنتجات لها درجة تشابه عاليه مع التركيب الجيني لجين الاي تي اس-٢ (ITS-2) من الإيميريا ستيدي لمعزولتي بني سويف/ مصر وإل واي/ الصين و تقع معها في نفس التصنيف الوراثي في شجرة التطور. أظهرت الدراسه ان النيستد بي سي أر لجين الأي تي إس-٢ (ITS-2) مفيد في التوصيف الجزيئي للإيميريا ستيدي في الأرانب.