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Original article

Morphological and molecular phylogenetic analyses of the apicomplexan parasites, *Eimeria media* and *Eimeria stiedai*, infecting the domestic rabbits, *Oryctolagus cuniculus*

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ABSTRACT

The eimerian infection is one of the most serious infections that can decrease rabbit productivity since it can lead to serious diseases. There is little information about *Eimeria media* and *Eimeria stiedai* infections in Saudi Arabia, and molecular data is particularly weak. To establish the prevalence, morphological and molecular characterization of *E. media* and *E. stiedai* isolated from spontaneously infected rabbits, the current study was conducted. Ten healthy rabbits, *Oryctolagus cuniculus*, whose feces were collected and kept at the laboratory were examined for protozoan parasite infection using the floatation method. Purified oocysts were used to extract DNA, which was then used in a polymerase chain reaction (PCR) with primers that amplified a partial sequences of the 18S rDNA gene. Seven rabbits had coinfections with two eimerian species. Sporulated oocysts of *E. media* had an obvious micropyle and were oval, measuring 24.92–30.03 (28.04) µm in length and 16.33–19.63 (18.31) µm in width. In contrast, *E. stiedai* were ellipsoid and measured 31.03–36.47 (33.79) µm in length and 18.21–20.93 (19.32) µm in width. The identity of the species of *Eimeria* parasites detected from the host (rabbits) was verified by the results of the sequences for the 18S rDNA gene. Both organisms (*E. media* and *E. stiedai*) grouped with rabbit eimerian parasites with distinct association with the group that contains oocysts residual body. Sequences from *E. stiedai* revealed insertions on two sites that had never been detected in *E. stiedai* sequences previously deposited in GenBank. The current parasite species are closely related to the previously described and deposited *E. media* and *E. stiedai* and are deeply embedded in the genus *Eimeria* (family Eimeriidae). This study emphasized the significance of combining taxonomy with morphological and genetic data in the identification of *Eimeria* species.

1. Introduction

Coccidiosis is caused by obligate intracellular apicomplexan protozoan parasites of the genus *Eimeria* (family Eimeriidae), which is one of the most common parasitic disease in domestic animals such as rabbits and chickens (Petrova et al., 2022). Some researchers have previously claimed that domestic rabbits in Saudi Arabia had *Eimeria* species (Kasim and Al-Shawa, 1987; Toula and Ramadan, 1998; Bashtar et al., 2003; Shazly et al., 2005; Al-Mathal, 2008; Al-Quraishy, 2012; Dkhil et al., 2013; Abdel-Baki and Al-Quraishy, 2013). The most common way for the vulnerable host to become infected is through consuming sporulated oocyst in contaminated food or water (Hamid et al., 2019). *Eimeria* species go through a complex monoxenous life cycle after endogenous (intra-host) development (merogony and gamogony) and sporogony in the environment (Petrova et al., 2022).

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In rabbits (*Oryctolagus cuniculus*), there are 15 recognized *Eimeria* species 14 species of which colonize the gastrointestinal tract (*E. intestinalis, E. magna, E. piriformis, E. media, E. exigua, E. flavescens, E. coecicola, E. vejdovskyi, E. roobroucki, E. perforans, E. oryctolagi, E. nagpurensis*, *E. agnotsa, E. irresidua,* and *E. matsubayashi*) while *Eimeria stiedai* is the 15th species inhabits the biliary ducts of the liver (Shil and Roy, 2021). Most eimerian species that infect rabbits affect production, depending on their pathogenicity, they may induce slower development, reduced feed conversion, and increased mortality rate (El-Shahawi et al., 2012). A normal fecal investigation usually reveals that a rabbit can have concurrent infections from multiple *Eimeria* species (Jithendran and Bhat, 1996).

According to several studies, morphological features, the time for sporulation, the affected site by infection, and signs of disease are the most frequently used criteria to identify *Eimeria* species (El-Shahawi et al., 2012). The various degrees of overlap among all these biological characteristics may occasionally make precise identification of *Eimeria* species problematic. The molecular characteristics of the *Eimeria* species have recently been determined using some genetic markers (Kvičerová et al., 2008). The 18S rDNA gene, one of the most prevalent loci, has been utilized to investigate the inter- and intra-species variation among *Eimeria* isolates, as well as their phylogenetic relationship (Ogedengbe et al., 2011). Furthermore, molecular identification approaches for *Eimeria* species from chickens and rabbits have been established using the internal transcribed spacer 1 (*ITS*-1) region (Su et al., 2003). Many apicomplexan parasites have the mitochondrial cytochrome oxidase (*COI*) gene locus, which has been extensively used for genotyping and species identification, including *Eimeria* species from turkey, ferret-badger and skink (Imai and Barta, 2019).

Even though rabbits are a significant animal protein source, there has been very little information on the parasitology of intestinal parasites (Atta et al., 1999; Abu-Akkada et al., 2010; Abdel-Gaber et al., 2019). In this investigation, rabbit-isolated *Eimeria media and Eimeria stiedai* were described morphologically and characterized molecularly using the partial 18S rDNA sequences.

2. Materials and methods

2.1. Animals collection

Ten domestic rabbits, *Oryctolagus cuniculus*, were collected from Riyadh's local markets (Saudi Arabia). Each Rabbit was maintained separately in wire-floored batteries at optimal conditions (temperature and humidity) and fed commercial rabbit feed (without the use of reference anticoccidial drugs). The institution's policies on the handling and use of animals in research (approval number KSU-SE-22–38) were followed in the care and rearing of the animals.

2.2. Parasitological examination

Using screens just below the cages, fresh fecal samples were taken, instantly excreted and then tested for *Eimeria* infection. Following the procedure outlined by Abdel-Baki and Al-Quraishy (2013), positive samples were collected and concentrated using the floatation technique. To allow for oocyst sporulation, the oocysts were collected and placed in 2.5% (w/v) potassium dichromate ($K_2Cr_2O_7$) and incubated for 5–7 days at 25 ± 2 °C. The sporulated oocysts were stored at 4 °C until they were used after being repeatedly washed in phosphate-buffered saline (PBS).

2.3. Species identification

Using the keys previously mentioned by Catchpole and Norton (1979), the oocysts were identified based on their size (length/width), morphological features, and internal structures. A calibrated micrometer was used to measure about 50 sporulated oocysts. All measurements were taken in micrometers (μm) and were shown as a range with mean between brackets.

3. Molecular methods

3.1. DNA extraction

Purified oocysts were subjected to DNA extraction using the method of Zhao et al. (2001) which included lysis buffer and Cetyl–Trimethyl Ammonium Bromide (CTAB) buffer (consisting of 2% w/v CTAB, 1.4 M NaCl, 0.2% β-mercaptoethanol, 20 mM EDTA, 100 mM TRIS), with minor modification by using 1.3% N-Sodium Dodecyl Sulphate (SDS) versus 1.3% N-lauroylsarcosine. The isolated oocysts were then treated with sodium hypochlorite (5–6%), and incubated for 45 min at 65 °C in the lysis buffer. The mixture was then incubated for a further one hour at 65 °C with 350 μl of CTAB buffer. Following the manufacturer's steps, sporulated oocysts were also used for DNA extraction using the Isolate II fecal DNA extraction kit (Meridian Bioscience, London, UK).

3.2. 18S rDNA amplification and sequencing

The partial 18S rDNA gene was amplified by PCR using the primers F1E 5′-TACCCAATGAAAACAGTTT-3′ as a forward primer and a reverse primer, R2B 5′-CAGGAGAAGCCAAGGTAGG-3′ (Orlandi et al., 2003). The Polymerase Chain Reaction (PCR) was set up in 25 μl using Bioline Buffer (Bioline, London, UK), which contained 200 μM concentrations each of dNTPs. The reaction mixture was adjusted to a final concentration of 2 mM $MgCl_2$ and 0.2 μM primer concentrations. The PCR mixture was supplemented with DNA Taq polymerase (Bioline). The PCRs were performed in a Multigene™ thermocycler (Labnet International, Inc., Edison, NJ, USA). As an initial denaturation cycle, the amplification program began with 2 min at 94 °C. The cycling conditions were denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 30 s. Before holding the PCR at 4 °C, a final extension at 72 °C for 5 min was permitted. The PCR products were separated by electrophoresis on a 1.5% agarose gel in 1 × Tris–boric acid–EDTA (TBE), stained with ethidium bromide and examined with a UV *trans*-illuminator. Macrogen's sequencing facility (Seoul, South Korea) was used for the DNA sequencing.

3.3. Phylogenetic analysis

The National Center for Biotechnology Information (NCBI) was used to do a BLAST search to find the relevant sequences. The CLUSTAL-X multiple sequence alignment with default parameters was used to directly align the sequences. The alignment that resulted was manually edited with the help of the application BioEdit 7.2.5. The 18S rDNA locus, which is available in the GenBank database, was used to infer phylogenetic relationships between rabbit isolates from related *Eimeria* species. The collection comprised representative sequences from some related Eimeriidae found in GenBank. Maximum likelihood (ML) and Neighbour Joining (NJ) analyses were carried out to infer the relationships among the 18S rDNA dataset using MEGA X (Kumar et al., 2018). As an outgroup, *Toxoplasma gondii* (LC 749847.1) was added. 1000 replicates were used to assess the bootstrap support in ML tree constructs.

4. Results

Out of 10 rabbits examined, 7 were infected with mixed *Eimeria* spp., indicating a prevalence of 70%. Two morphologically distinct eimerian oocysts were detected in the fecal material, which was related to *Eimeria media* and *Eimeria stiedai*. *E. media* was the most predominant species, while infection with *E. stiedai* was less common. The oocysts of *Eimeria* species in the present study were illustrated in Figs. 1 and 2. Tables 1 and 2 summarize the morphological and morphometric characteristics of the *Eimeria* species.

4.1. ***Description of Eimeria media*** *(Fig. 1 and Table 1)*

Sporulated oocysts are ellipsoid and measured 24.92–30.03 (28.04) μm in length and 16.33–19.63 (18.31) μm in width, with a noticeable micropyle. The oocyst index (length/width) was 1.53. The wall of the oocyst is double-layered, with a smooth outer layer, somewhat thickened, forming a small ridge at the micropyle, and a membranous inner layer. The oocyst residuum is rounded, and the polar granule is absent. Each oocyst contained four dizoic sporocysts with a pointed end, measuring 10.22–15.18 (13.09) μm in length and 4.34–6.57 (5.47) μm in width. They were ellipsoid and surrounded by a single-layer sporocyst wall. The stieda body and residuum were present. The sporocyst index (length/width) was 2.39. One refractile body is located at the wider end of each sporozoite.

4.2. ***Description of Eimeria stiedai*** *(Fig. 2 and Table 2)*

Sporulated oocysts are ovoid and measured 31.03–36.47 (33.79) μm in length and 18.21–20.93 (19.32) μm in width, with an observable micropyle. Absence of both oocyst residuum and the polar granule. There are four sporocysts (each with two elongated sporozoites). Each sporocyst is ovoid and measured 11.18–15.30 (13.38) μm in length and 5.68–8.60 (6.94) μm in width. The stieda body is present. Absence of both substieda and parastieda bodies. Granular sporocyst residuum present.

4.3. Molecular analysis

A PCR product of ~636 bp was successfully amplified and sequenced from the 18S rDNA gene of eimerian isolates. PCR products from two isolates of *E. stiedai* produced identical sequences for the 18S rDNA whereas one isolate from *E. media* was amplified and sequenced. Sequences of *E. stiedai* were given GenBank accession numbers OQ704326 and OQ704327 and sequence from *E. media* was given GenBank accession number OQ704328. The final alignment of 611 bp of the 18S rDNA was used for the phylogenetic analysis which included 24 sequences with *Toxoplasma gondii* as an outgroup. Phylogenetic analyses showed two distinct clades for *Eimeria* species infecting rabbits (Fig. 3). Both *E. stiedai* and *E. media* detected in this study clustered with the clade which contained *E. intestinalis, E. media, E. perforans, E. coecicola, E. vejdovskyi,* and *E. stiedai*. Another clade grouped other rabbit eimerian parasites including *E. exigua* and *E. piriformis*. However, within this clade, *E. stiedai* formed a subclade that distinguished *E. stiedai*. There are only two 18S rDNA sequences related to *E. stiedai* in GenBank (EF694008 and HQ173837) and both were from the Czech Republic. Both sequences were homologous to the recovered sequences. However, the recovered sequences showed significant differences from those other sequences of *E. stiedai*. Two insertions of 4 and 6 nucleotides at positions 271 and 305 respectively were noted in sequences obtained in the present study. The insertion at position 271 (3 bases) was found in the sequences EF694008 whereas it was not found in sequences HQ173837. The other inser-

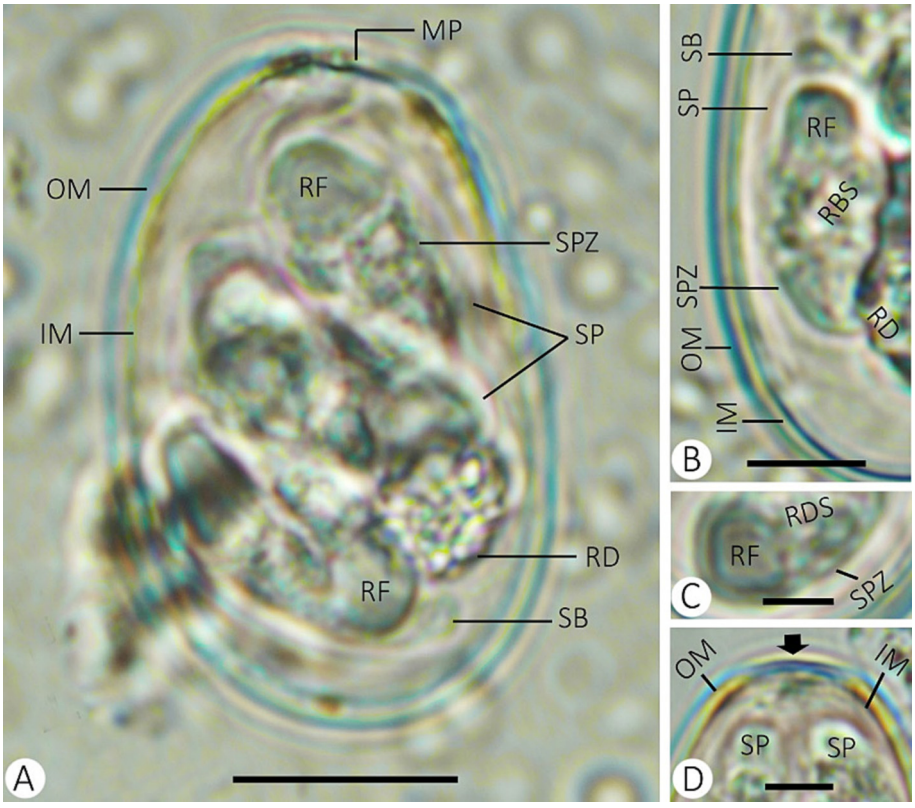

Fig. 1. *Eimeria media* infecting rabbis. (A) sporulated oocyst. (B) sporocyst with sporozoites. (C) refractile body of sporozoite. (D) micropyle of the oocyst. (Note: MP, micropyle; OM, outer membrane; IM, inner membrane; RF, refractile body; RD, residuum; SB, stieda body; SP, sporocyst; RDS, residuum of sporocyst; SPZ, sporozoite) Scale = 10 μm.

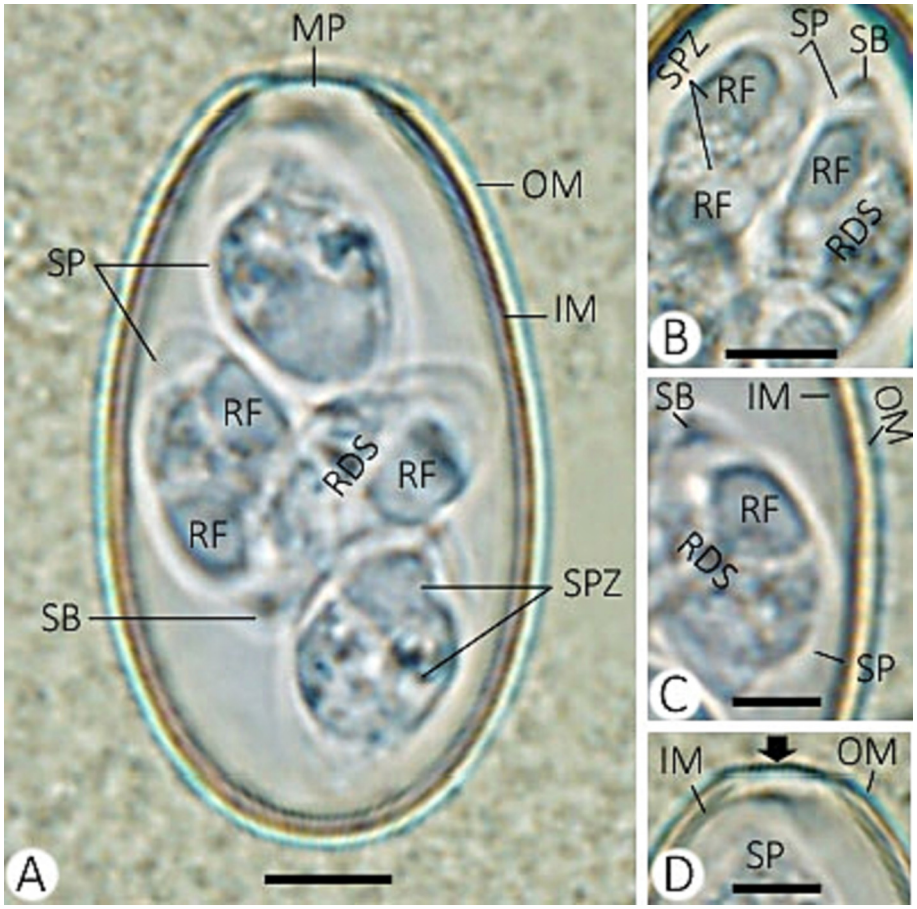

Fig. 2. *Eimeria stiedai* infecting rabbis. (A) sporulated oocyst. (B) sporocysts with sporozoites. (C) refractile and stieda bodies of sporozoite. (D) micropyle of the oocyst. (Note: MP, micropyle; OM, outer membrane; IM, inner membrane; RF, refractile body; SB, stieda body; SP, sporocyst; RDS, residuum of sporocyst; SPZ, sporozoite) Scale = 10 μm.

Table 1
Morphological characteristics of sporulated oocysts for *Eimeria media* from rabbits.

Sources of *E. media* species	Shape	Oocyst size		Micropyle	Oocyst residuum	Sporocyst size		Locality
		Length	Width			Length	Width	
Kheyssin, 1967	–	18.5–33.3	13.3–21.3	–	–	6.6–14.6	5–7	–
Levine and Ivens, 1972	–	19–37	13–22	–	–	17.5	7	–
Pakandl, 1986	Ellipsoid (Type I)	24–33 (29.05)	15–20 (18.18)	+	+	14–17 (15.79)	6–8 (6.67)	Ceské Budějovice
Pakandl, 1986	Ellipsoid (Type II)	30–37 (32.88)	17–21 (19.19)	+	+	14–16.5 (15.71)	7–9 (8)	Ceské Budějovice
Kasim and Al Shawa, 1987	Ellipsoid-ovoid	25.5–34 (30)	15–22 (18.7)	+	+	9–16 (12.9)	5–8.5 (6.6)	Saudi Arabia
Li et al., 2010	–	21.8–34.6 (29.3)	16.0–21.3 (18.3)	+	+	8.0–17.3 (13.8)	5.3–10.6 (7.6)	Taiwan
El-Shahawy et al., 2012	Ellipsoid-ovoid	19–24 (22.3)	10–15 (12.1)	+	+	7–9 (8.2)	4–6 (4.5)	Egypt
Abdel-Baki and Al Quraishy, 2013	Ellipsoid	19–24 (22)	10–15 (12)	+	+	7–9 (8)	4–6 (4.5)	Saudi Arabia
El-Shahawy and Elgoniemy, 2018	Ellipsoid-ovoid	28.64	16.70	+	+	12.17	7.34	Egypt
El-Sayed et al., 2020	Ellipsoid-ovoid	27.48	17.79	+	+	10.60	6.42	Egypt
Rabie et al., 2022	Ellipsoid	25.34–29.4 (27.44)	16.36–22.11 (18.61)	+	+	11.22–15.88 (13.72)	6.5–8.01 (6.95)	Egypt
Present study	Ellipsoid	24.92–30.03 (28.04)	16.33–19.63 (18.31)	+	+	10.22–15.18 (13.09)	4.34–6.57 (5.47)	Saudi Arabia

+ present, - not detected.

tion of 6 bases was missing in sequences HQ173837 and only three bases were missing in sequences EF694008. The sequence of *E. media* (OQ704328) grouped was homologous to the sequence from *E. media* (HQ173834) from France with two mutations at positions 174 and 487 of the alignment and both changes transversions T/A and T/G respectively.

5. Discussion

Due to the related morbidity and mortality, parasitic infections pose a serious effect on human health as well as a detrimental effect on livestock productivity. The prevalence of parasitic diseases specifically has a detrimental impact on rabbit production

Table 2
Morphological characteristics of sporulated oocysts for *Eimeria stiedai* from rabbits.

Sources of *E. stiedai* species	Shape	Oocyst size		Micropyle	Oocyst residuum	Sporocyst size		Locality
		Length	Width			Length	Width	
Aoutil et al., 2005	Ellipsoid	33–40 (37)	19–24 (22)	+	–	18	10	France
Al-Mathal, 2008	Oval	30	10–20	–	–	10	–	Saudi Arabia
El-Shahawy et al., 2012	Ellipsoid	24–29 (26.5)	11–15 (13.1)	+	+	9–11 (9.7)	5–6 (5.6)	Egypt
Abdel-Baki and Al-Quraishy, 2013	Ellipsoid	25–29 (26)	12–15 (13)	+	+	9–11 (10)	5–7 (6)	Saudi Arabia
Ütük et al., 2015	–	34–38 (36.1)	17–21 (19.7)	+	–	12–17 (15.0)	8–10 (8.9)	Turkey
Rabie et al., 2022	Ovoid	27.65–28.44 (28.13)	16.55–17.66 (16.99)	+	–	10.82–11.18 (11.01)	5.67–6.03 (5.89)	Egypt
Present study	Ovoid	31.03–36.47 (33.79)	18.21–20.93 (19.32)	+	–	11.18–15.30 (13.38)	5.68–8.60 (6.94)	Saudi Arabia

+ present, - not detected.

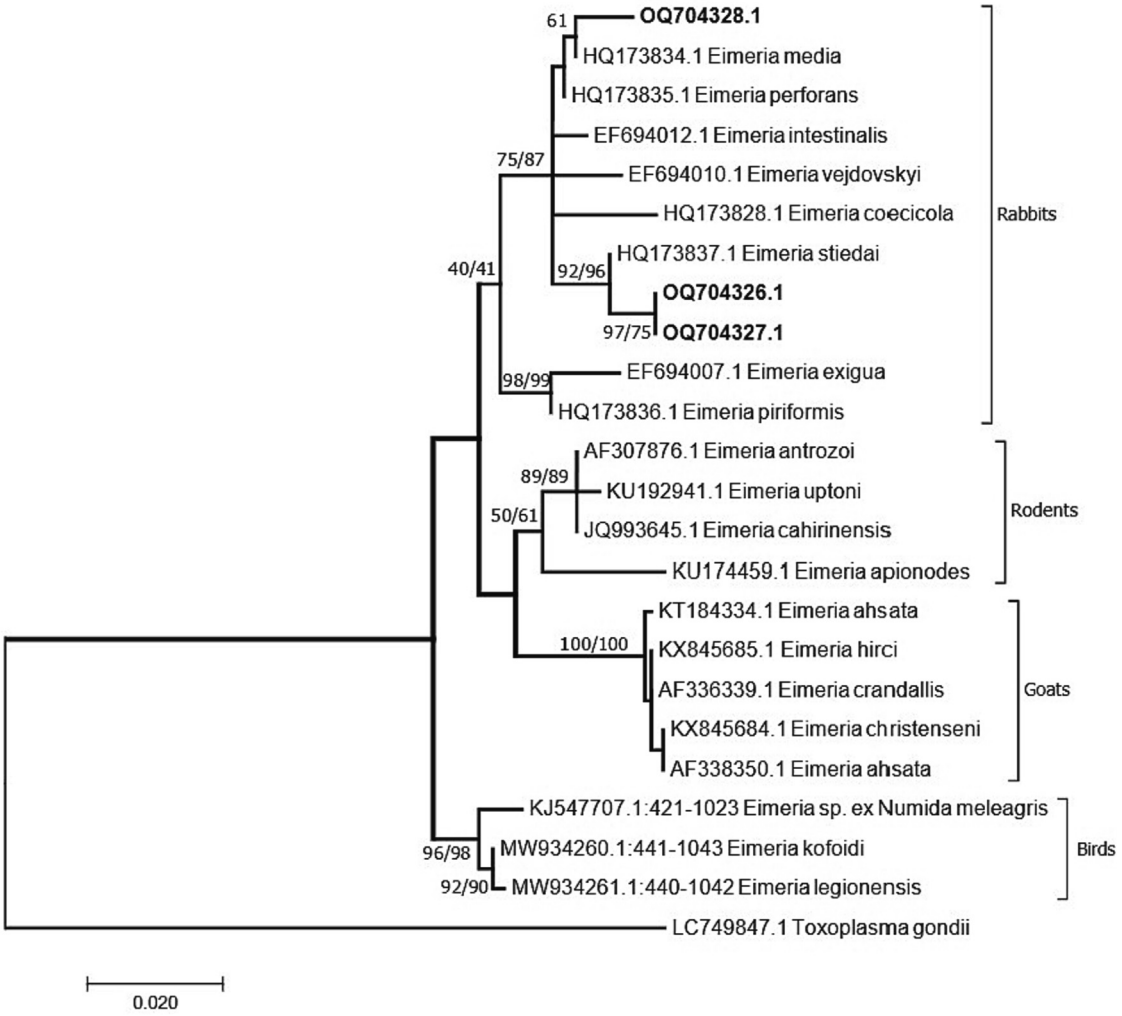

Fig. 3. A consensus phylogenetic tree constructed with maximum likelihood (ML) and Neigbour Joining (NJ) methods, showing phylogenetic relationships among *Eimeria media* and *E. stiedai* and other eimerian coccidia, with *Toxoplasma gondii* (Nicolle et Manceaux, 1908), as an outgroup, inferred from the 18S rDNA sequence data generated from the two eimerian species from rabbits (OQ704326-704328, given in bold) and other related taxa from GenBank. Numbers indicated at branch nodes are bootstrap values (ML/NJ). Only bootstraps > 40% are shown.

and causes a large economic loss (Abdel-Baki and Al-Quraishy, 2013). Therefore, gaining an awareness of intestinal parasite species can help with minimizing the economic rabbit productivity, determining the infection risk, and developing management strategies. According to the available literature, Saudi Arabia has conducted very little research on *Eimeria* of rabbits (Kasim and Al-Shawa, 1987; Al-Mathal, 2008; Abdel-Baki and Al-Quraishy, 2013). In this study, we attempted to integrate morphological and genetic information on the rabbit-infecting *Eimeria* species.

In the current study, a significant number of rabbits (70%) had an *Eimeria* species infection. In earlier studies on rabbits, similar trends were documented (Li et al., 2010; Abdel-Baki and Al-

Quraishy, 2013). Herein, mixed species of *Eimeria* infections were found, which is consistent with reports of El-Shahawy and Elgoniemy (2018) and Rabie et al. (2022) that single *Eimeria* species infections naturally occur infrequently. This may be the case since some parasite species may be found in food. *Eimeria* species are distinguished by their oocyst and sporocyst size, morphological features, prepatent duration, and colonization site (El-Sayed et al., 2020). In the feces of the rabbits obtained from Saudi Arabia for the current study, two species of *Eimeria*, *E. media,* and *E. stiedai*, were identified according to these criteria.

The descriptions of the sporulated *E. media* oocysts are basically in line with what Rabie et al. (2022) reported in their earlier work. These species do, however, differ slightly from earlier descriptions in terms of sporocyst and oocyst size as well as a few other negligible characteristics. Here are a few minor observations: (i) compare to previous studies by El-Shahawi et al. (2012) and Abdel-Baki and Al-Quraishy (2013), the oocysts and sporocysts of *E. media* in the present study are larger. (ii) the oocyst shape was ellipsoid, except for those identified as ovoidal to ellipsoidal by Kasim and Al-Shawa (1987), El-Shahawi et al. (2012), El-Shahawy and Elgoniemy (2018), and El-Sayed et al. (2020). (iii) The specimens described by Kheyssin (1967) and Levine and Ivens (1972) lacked micropyle and oocyst residuum of *E. media.*

Our study's *E. stiedai* sporulated oocysts are generally consistent with those from Ütük et al. (2015). The following findings can be drawn when contrasting this *Eimeria* species to other species that have been described: (i) Compared to previous studies by El-Shahawy et al. (2012), Abdel-Baki and Al-Quraishy (2013) and Rabie et al. (2022), the oocysts and sporocysts of *E. stiedai* in the present study are larger. (ii) All oocysts were ovoid, with the exception of those mentioned as ellipsoid oocysts by Aoutil et al. (2005), El-Shahawy et al. (2012), and Abdel-Baki and Al-Quraishy (2013). (iii) The specimens described by Al-Mathal (2008) lacked the micropyle of *E. stiedai.* (iv) Other than in El-Shahawy et al. (2012), and Abdel-Baki and Al-Quraishy (2013), the oocyst residuum of *E. stiedai* was lacking.

Oocyst morphology can be used to distinguish between *Eimeria* species, however, it is time-consuming and labor-intensive. At the species level, it is extremely difficult to identify, especially in mixed infections where there aren't enough oocysts of a particular species. The proper identification of *Eimeria* species has recently benefited from molecular methods (Ütük et al., 2015; Al Quraishy et al., 2022).

Molecularly, based on the 18S rDNA analysis, sequences from both oocysts detected in this study clustered with the clade of eimerian parasites infecting rabbits. However, two distinct clades were found within eimerian species of rabbits. The first one included *E. intestinalis, E. perforans, E. media, E. coecicola, E. vejdovskyi,* and *E. stiedai* whereas the other one included *E. exigua* and *E. piriformis*. The first group of eimerian parasites included those which possess oocyst residual body whereas the other group included those without oocyst residual body. This finding coincided with the results outlined by Kvičerová et al. (2008). Within those included in the clade with oocysts residual body, *E. stiedai* formed a distinct subclade that distinguished it from all other species in the group. It is likely this because *E. stiedai* inhabits extraintestinal locations where it occurs in the bile ducts, furthermore, it has low host specificity, unlike other eimerian parasites from rabbits which have been reported from the hare (Scholtyseck et al., 1979). Molecular characterization confirmed the identity of the oocysts detected in the present study. However, there was wide variation in the lengths of the size of *E. media* oocysts ranging from 18 to 37 μm in length and 10–22 μm in width. Molecular data have confirmed the identity of the oocysts detected herein, therefore, it is very important to have the molecular data alongside morphological data to identify eimerian parasites adequately.

Ethical approval

This research was approved by the Research Ethics Committee (REC) at King Saud University (approval number KSU-SU-22–38).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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