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ORIGINAL ARTICLE

ISOLATION AND IDENTIFICATION OF *EIMERIA* SCHNEIDER, 1875 SPECIES (APICOMPLEXA, EIMERIIDAE) FROM GOATS IN WASIT PROVINCE, IRAQ

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ABSTRACT

Eimeriosis is a major problem affecting ruminants worldwide. The disease is primarily caused by *Eimeria* species, which are specialized for each host and grow in the small and large intestine of animals. The losses due to subclinical infections (especially weight loss) and clinical disease (diarrhea) make the species of this genus a very significant economic concern. Therefore, this study was conducted in some areas of Wasit Province. A total of 180 fecal samples from goats, of both sexes and covering different age groups and months, were collected. All fecal samples were examined microscopically, and 75 positive fecal samples were taken for molecular examination and further analyzed using conventional PCR, sequencing and phylogenetic analysis. Microscopic results showed that the overall infection rate was 41.6%. The incidence of *Eimeria* species ranged from 5.55% to 22.22% across three different species of the genus *Emeria* Schneider, 1875, namely *E. arloingi* (Marotel, 1905), Martin, 1909 (22.22%), *E. christenseni* Levine, Ivens & Fritz, 1962 (13.88%), and *E. hirci* Chevalier, 1966 (5.55%). Regarding the PCR reaction, results from the 18S rRNA, COI gene and genetic sequencing, Confirmed that the fecal samples were positive for *Eimeria* Schneider, 1875 species.

Keywords: COI gene, Eimeriosis, *Eimeria* species, Goat, 18S rRNA, Wasit Province.

INTRODUCTION

Intestinal infection of goats has been associated with a species of protozoan that belongs to the genus *Eimeria* Schneider, 1875 (Apicomplexa: Eimeriidae) (Mohamaden *et al.*, 2018). Any stage of an animal's life may experience mixed infection with pathogenic and/or nonpathogenic species, with younger animals being more vulnerable (Keeton and Navarre, 2018; Smith *et al.*, 2019).

According to Chartier and Paraud (2012), Eimerosis, one of the most significant parasite diseases affecting goats, has spread around the globe. Due to high mortality and morbidity rates, indigent development and costly treatments, this condition results in substantial economic losses (Temizel *et al.*, 2011). This parasite is transmitted via mature oocysts,

which require specific environmental conditions, such as humidity and temperature, to develop and transform into sporozoite- containing structures (Malla and Saida, 2022).

According to Rehman *et al.* (2011) and Cavalcante *et al.* (2012), the cause of eimeriosis is an intestinal protozoan parasite belonging to the genus *Eimeria*. Seventeen species of *Eimeria* have been identified in goats worldwide. Among these, *E. christenseni* Levine, Ivens & Fritz, 1962, *E. arloingi* (Marotel, 1905) Martin, 1909, *E. caprina*, and *E. ninakohlyakimovae* Yakimoff & Rastegaieff, 1930, are considered significant infections (Silva and Lima, 1998; Rajakaruna *et al.*, 2012). Young animals under the age of four as well as animals of any age maintained in overcrowded conditions with added stressors are more severely affected. Examples of such stressors include weaning, nutritional adjustments, travel, temperature extremes, and so forth (Gul, 2007). Infected animals excrete oocysts in their faeces (Temizel *et al.*, 2011). By ingesting sporulated oocysts, goats become infected. As sporulated oocysts enter intestinal epithelial cells in the small intestine, they release sporozoites causing electrolyte and nutritional malabsorption (Temizel *et al.*, 2011; Zachary and McGavin, 2014). *Eimeria* species was diagnosed using a variety of techniques, including molecular, serological, and fecal testing (Woods *et al*., 2000). Molecular methods are particularly sensitive and specific for fecal analyses (Sweeny *et al*., 2011). *Eimeria* has been diagnosed by PCR, which amplifies DNA, in a various hosts, providing reliable results across a range of samples (Kawahara *et al*., 2010).

The aim of the study was to identify the species of *Eimeria* infecting goats in Wasit Province using genetic sequencing.

MATERIALS AND METHODS

Sampling: In Wasit province, 180 fecal samples were collected from goats (83♂♂ and 97♀♀) aged between 5 months and over 6 years, from three sites: Al-Suwaira, Al-Aziziyah, and Al- Shaihemiyh Districts, with 60 fecal samples were collected from each site. From November 2022 to April 2023, fecal fecal samples weighing between 10-20 g were randomly collected from various farms. Immediately, while donning gloves, the samples were collected directly from the rectum and preserved at 4 °C until analysis (Ekawasti *et al.*, 2021).

Microscopic examination: After collection, the fecal samples were examined at the vertebrate laboratory of the Iraq Natural History Research Center and Museum, University of Baghdad, for further testing. These include direct smear, flotation in saturated salt solution, and culture with 2.5% Potassium Dichromate (wt./vol.)] and put in screw-cupped plastic containers. A microscope was used to identify the different species of *Eimeria* by using 40X power (Eckert *et al.*, 1995).

Molecular study

DNA extraction: DNA was extracted from 75 fecal samples which were collected and stored previously at -20ºC using DNA extraction kit /Korea.

Primers: The primers used for PCR amplification were designed according to Albanese *et al*. (2019) based on 18S rRNA with Primer sequence F (5'-CGCGCAAATTACCCAATGAA- 3'), R (5'- ATGCCCCCAACTGTCCCTAT- 3') gene at 454 bp and Bawn *et al.* (2020) on COI (cytochrome oxidase subunit I) gene sequences with Primer sequence F (5′-AGGTGTTGCTAATGGAGCCAA-3′), R (5′ ACAGCTGAGAAGTATGCTCTGG-3′) at 405 bp.

PCR reaction preparation: PCR reaction was performed using Master mix (Promega/USA) Component, including Master Mix or GoTaq® Green Master Mix (12.5µl), Forward primer (10 picomols/µl (1 µl), Reverse primer (10 picomols/µl (1 µl), DNA (1.5µl) and Distill water (9µl).

PCR thermocycler conditions: The thermocycler conditions for PCR were conducted usinga conventional PCR thermocycler system. For the 18S rRNA gene, the conditions included: Initial Denaturation (Temperature 95 °C for 5 min with 1 cycle), Denaturation 2 (Temperature 95 °C for 45 sec), Annealing (Temperature 56 °C for 45 sec), Extension 1 (Temperature 72 °C for 1 min) with 35 cycle and Extension 2 (Temperature 72 °C for 5 min with 1 cycle). While COI gene was conducted using conventional PCR thermocycler system which included Initial Denaturation (Temperature 94 °C for 3 min with 1 cycle), Denaturation 2 (Temperature 94 °C for 45 sec), Annealing (Temperature 58 °C for 30 sec), Extension 1 (Temperature 72 °C for 1 min) with 35 cycle and Extension 2 (Temperature 72 °C for 7 min with 1 cycle).

Sequencing of DNA and phylogenetic tree: Genetic sequencing was performed on seven positive samples in order to identify *Eimeria* species at the molecular level. The UPGMA (unweighted pair group method with arithmetic mean) approach was used to infer the evolutionary history. To obtain the bioinformatics for local goat *Eimeria* and to assign accession numbers for local *Eimeria* species in Iraq, a phylogenetic tree and the Genbank database were used. The 18s rRNA PCR product and the COI gene were shipped from the Korean company Macrogen. Once the sequences of the *Eimeria* species were acquired, they were submitted to the NCBI-GenBank to obtain Genbank accession numbers. Molecular Evolutionary Genetics Analysis (MEGA6) and multiple sequence alignment based on Clustal W alignment analysis were used for the DNA sequencing study. This includes phylogenetic tree analysis and multiple sequence alignment which were conducted using Clustal W alignment analysis and molecular evolutionary genetics analysis (MEGA6). Known sequences from NCBI-Blast were used as references for comparison was to identify *Eimeria* species through phylogenetic analysis.

RESULTS

This research was carried out in Wasit Province with no significant difference among the areas studied, a total of 180 fecal samples 75 (41.6%) of the analyzed were positive for *Eimeria* oocysts. A significant association (p=0.013) was observed between November 2022 and April 2023. Both gender (83♂♂ and 97♀♀) showed infection rates that varied from 22.22 to 5.55% across various locations, with males having a higher infection rate than

females (48.1% and 36%) respectively, though the difference was not statistically significant. Goat fecal matter was used to identify three distinct *Eimeria* species based on their morphological characteristics in addition to the measurements (length and width) of oocyst, using a microscope ruler (ocular stage). The species were identified based on oocyst size, appearance, the presence or absence of micropyle, and the structure of the oocyst wall. The identified species included *E. arloingi* (22.22%), *E. christenseni* (13.88%) and *E. hirci* (5.55%) with the differences being highly statistically significant (Pls. 1-3; Tabs. 1-4).

Table (1): Microscopic examination with infection rate *Eimeria* species in goats concerning the area.

Area	Number of Samples Analyzed.	Number of positive	(%)	P value
Al-Suwaira	60	20	33.3	0.18
Al-Aziziyah	60	25	41.6	
Al- Shaihemiyh	60	30	50.0	
Total	180	75	41.6	

[The results of chi square analysis revealed that there was no significant association (p=0.18) between the area].

Table (2): The infection rate with *Eimeria* species according to months of study.

Months/2022 and 2023	Number of Samples Analyzed.	Number of positive	(%)	P value
November	30	10	33.3	
December	30	13	43.3	
January	30	15	50.0	
February	30	20	66.6	0. 013
March	30	10	33.3	
April	30	7	23.3	
Total	180	75	41.6	

[The results showed a statistically significant association (p=0.013) between the month of infection or sample collection and the rate of infection with February being the month of higher percentage of infection compared to others (66.6 % positive cases)].

Table (3): The infection rate with *Eimeria* species according to goat's gender.

Gender	Number of samples analyzed.	Number of positive	P value
Male	83	40 (48.1)	0.10
Female	97	35 (36)	
Total	180	75 (41.6)	

[The results of chi square analysis revealed that there was no significant association (p=0.10) between gender and the rate of infection].

Table (4): Total infection rate with morphological characteristics of different *Eimeria* species in goats.

Species	Number of samples analyzed	Number of positive	%	P value	Oocysts morphology	Length × Width (µm) Observed (Min-max)
E. arloingi	180	40	22.22	≤0.001	Micropyle presents with a prominent cap	33 * 15
E. Christensen		25	13.88		Micropyle presents with a prominent cap	41*23
E. hirci		10	5.55		Micropyle present with cap	22*18

[The results showed a very highly statistically significant association (p ≤0.001) among the species of the parasite and the infection rate with *E. arloingi* constituting the high percentage of infection (22.22 %) of all].

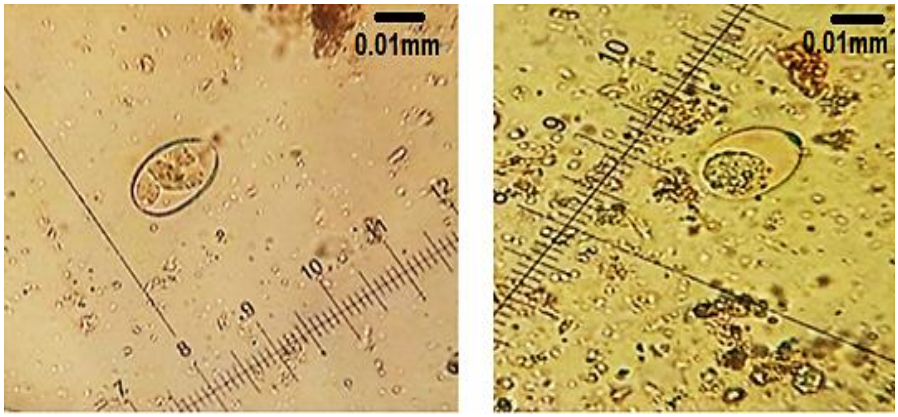

Plate (1): *Eimeria arloingi* (left sporulated and right unsporulated). (Oocysts are elliptic in form, have thick walls, and are coloured yellowish brown. They also have polar and micropyle caps).

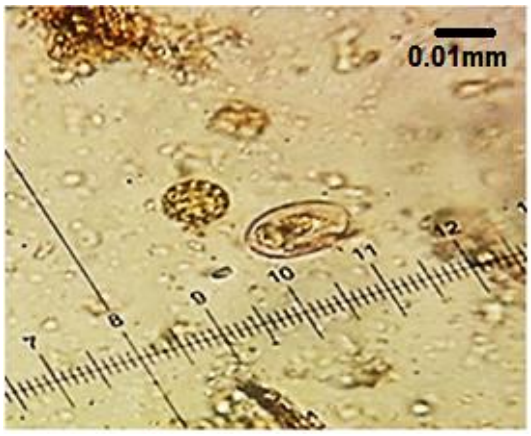

Plate (2): *Eimeria hirci* (with a micropyle and micropyle cap, oocysts are light brown to brownish yellow in morphology and ellipsoidal to subspherical in shape).

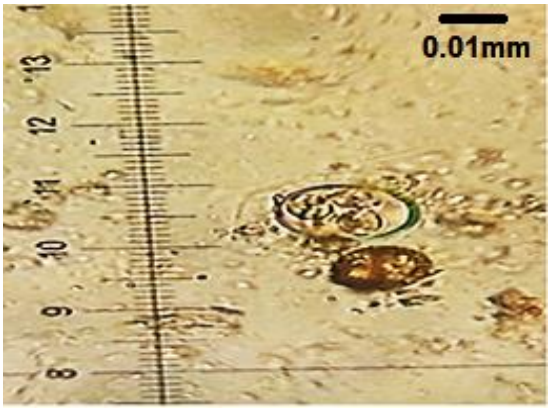

Plate (3): *Eimeria christensen* (Pear-shaped oocyst with a thick wall, micropyle cap, and polar cap; yellowish brown in tone).

Molecular results

Results obtained by PCR analysis: Seventy five fecal samples, which were examined traditionally in the lab, where further subjected to molecular examination. The samples tested positive result for *Eimeria* species using the 18S rRNA and COI gene (amplicons size H 454 bp and 405 bp) through the conventional PCR technique.

DNA sequencing results: Seven *Eimeria* positive samples were selected from the 75 positive samples for sequencing and phylogenic analysis. Sequenced samples were analysed using MegaX software and Multiple Alignments were created. The positive isolates were compared with NCBI isolates and the percentage of similarity was obtained. The sequences were then submitted to the NCBI-GenBank to acquire accession number codes for local *Eimeria* species in goat. The results revealed that two species were diagnosed by targeting

the 18S rRNA gene as *E. arloingi* (accession no. : PP862861.1, : PP862862.1, : PP862863.1, : PP862864.1) and *Eimeria christenseni* (accession no. PP862865.1, PP862866.1) (Tab.5), while the results revealed that three species were diagnosed by targeting the COI gene as *Eimeria hirci* (accession no. PP873185.1), *E. arloingi* (accession no. PP873186.1, PP873187.1, PP873188.1, PP873189.1) and *Eimeria christenseni* (accession no. PP873190.1, PP873191.1) (Tab. 6).

Table (5): The NCBI-BLAST homology sequence identity (%) between local *Eimeria* isolates from goat's faeces that were deposited in 18S rRNA gene bank and NCBI-BLAST deposited strains.

No.	Accession No	Identical to	Genbank accession No.	Country	Identity %
1	ID: PP862861.1	*E. arloingi*	ID: ON259586.1	China:Shaanxi	100%
2	ID: PP862862.1	*E. arloingi*	ID: ON259586.1	China:Shaanxi	99%
3	ID: PP862863.1	*E. arloingi*	ID: ON259586.1	China:Shaanxi	99%
4	ID: PP862864.1	*E. arloingi*	ID: ON259586.1	China:Shaanxi	99%
5	ID: PP862865.1	*E. christenseni*	ID: ON259585.1	China:Shaanxi	99%
6	ID: PP862866.1	*E. christenseni*	ID: ON259585.1	China:Shaanxi	100%

Table (6): The NCBI-BLAST homology sequence identity (%) between local Eimeria isolates from goat's faeces that were deposited in COI gene bank and NCBI-BLAST deposited strains.

No	Accession No	Identical to	Genbank accession No.	Country	Identity %
1	ID: PP873185.1	*Eimeria hirci*	ID: LC508122.1	Japan	100
2	ID: PP873186.1	*E. arloingi*	ID: LC508123.1	Japan	99
3	ID: PP873187.1	*E. arloingi*	ID: LC508123.1	Japan	100
4	ID: PP873188.1	*E. arloingi*	ID: LC508123.1	Japan	99
5	ID: PP873189.1	*E. arloingi*	ID: LC508123.1	Japan	100
6	ID: PP873190.1	*E. christenseni*	ID: LC508121.1	Japan	100
7	ID: PP873191.1	*E. christenseni*	ID: LC508121.1	Japan	100

Phylogenetic analysis

Phylogenetic tree analysis of *Eimeria* species based on the currently identified sequences (18S ribosomal RNA and COI gene) is represented with coloured triangle and their corresponding accession numbers. As it can be seen, the Iraqi strains mainly appeared closely related to each other. However, when targetingthe18S ribosomal RNA diagnosed *E. arloingi* and *Eimeria christenseni* were found to be closely related to an isolates from Shaanxi, China (Diags. 1, 2). Meanwhile, targeting the COI gene diagnosed *Eimeria hirci*, *E. arloingi*, and *Eimeria christenseni* as being similar to an isolate from Japan (Diags. 3, 4).

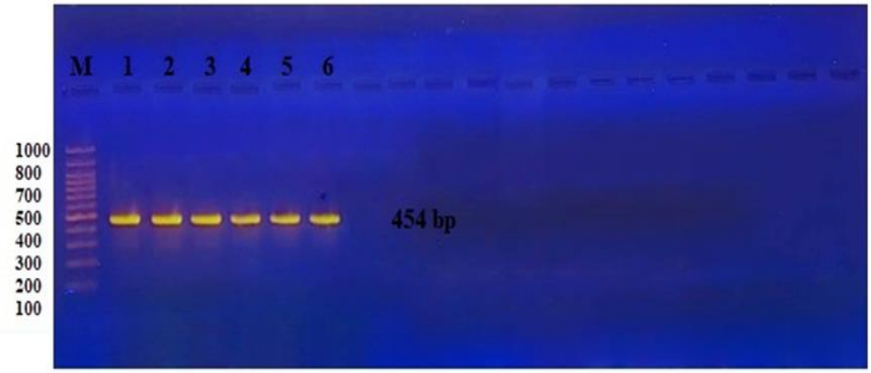

Diagram (1): Gel electrophoresis image (1% agarose) shows the positive samples of *Eimeria* spp. (amplicons size H – 454 bp) by targeting 18S rRNA gene.

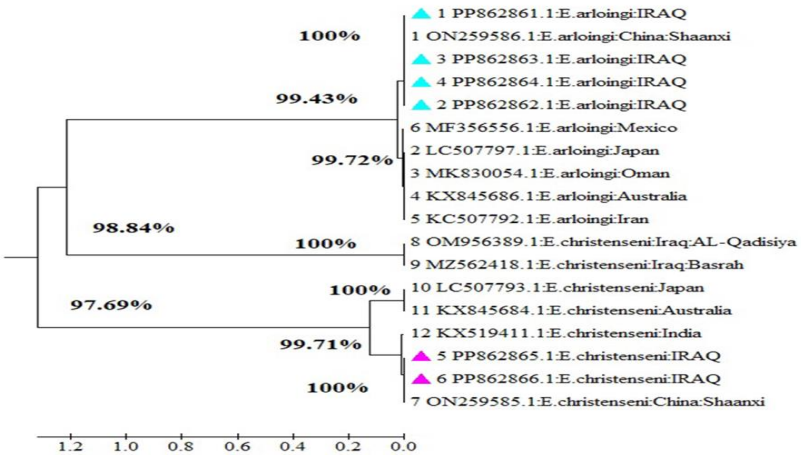

Diagram (2): Phylogenetic tree analysis for *Eimeria* species by targeting 18S rRNA gene

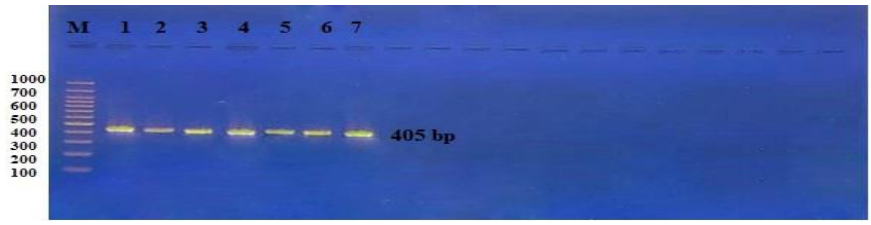

Diagram (3): Gel electrophoresis image (1% agarose) shows the positive samples of *Eimeria* species (amplicons size H – 405 bp) by targeting COI gene.

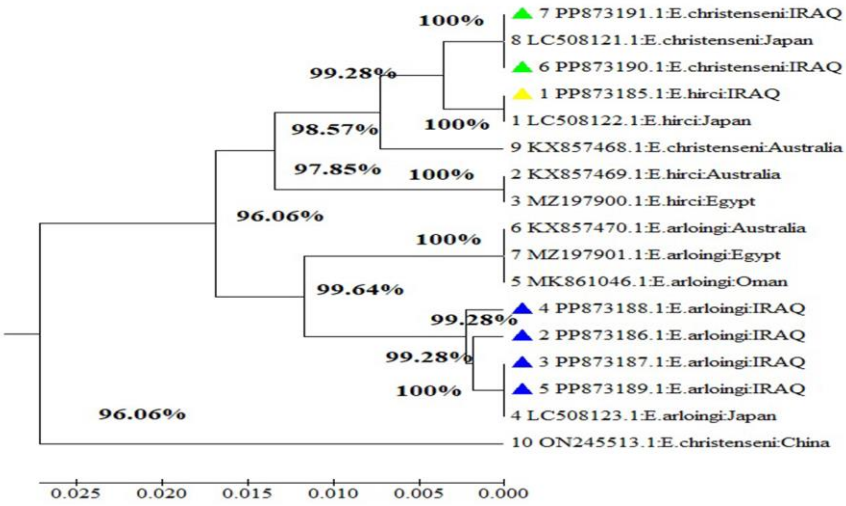

Diagram (4): Phylogenetic tree analysis for *Eimeria* spp. by targeting COI gene.

DISCUSSION

According to the findings, a variety of *Eimeria* species, including single, double, and multiple infections, parasitize goats residing in this area. The overall infection frequency was found to be 75 (41.6%) across three regions of the Waist province including, Al-Suwaira, Al-Aziziyah, and Al- Shaihemiyh, and there was no significant difference observed among areas. This study's high infection rate reflects unsanitary living Conditions, which are likely a contributing cause to the spread of eimeriosis, and grazing in exposed, contaminated places. According to the infection rate of *Eimeria* parasite, this study disagrees with that of Macedo *et al*. (2019) who reported infection rates of *Eimeria* spp. at 73.91% and 60% in goats. While Hasan and Mahmood (2021) recorded a subclinical eimeriosis rate of 69.05% in goats. Senbeto and Chekol (2022), who found that infection with *Eimeria* species was 50.5% in goats.

In addition, several studies have documented high infection rates (57.5%, 68.4%, 58%, 82.03%) in small ruminants (El-Alfy *et al.*, 2020; Ayana *et al.*, 2022; Carneiro *et al*., 2022), in Iraq, Egypt, Ethiopia and Brazil respectively. Higher prevalence rates were recorded in Vietnam 86.85%, India 90.96%, and Myanmar 60.0% (Bawn *et al.*, 2020; Singh *et al.*,

2020; Nguyen-Ho-Bao *et al.*, 2022). The present study's prevalence rates of *Eimeria* spp. oocysts in goats are lower than those previously reported. Eser *et al.* (2020) recorded a much lower infection rate as being 20.33% in Turkey. In the current study, the highest infection rates were recorded in February 66.6% and January 50, 66.6, and 50%, with a significant association (p=0.013) between infection rate and month. This finding aligns with the observation of Ibrahim (2012) who reported that the infection rate was higher during the rainy season (60.22%) compared to the dry season (45.81%). It is also consistent with Minnat (2014), who recorded a hight infection rate in February with a rate (100%). The increase in rainfall, humidity, and fall in temperature during February and January may attributive the higher infection rates of *Eimeria* sp. (Saleh, 2011). Regarding gender, no significant difference was observed in the current study. Similarly, Ibrahim (2012) observed a slightly higher infection rate in males (55.19%) compared to females (51.63%). Additionally, Verma *et al.* (2018) also discovered that the rates of infection in males were greater than that in females, 71.55% and 71.39% respectively. Male animals may be more susceptible to infections due to the immunosuppressive effects of high androgen levels, particularly testosterone, in their plasma during the reproductive season (Souza *et al*., 2015).

The mentioned species were diagnosed based on the measurements described by Taylor *et al*. (2007) and Verma *et al*. (2018). Numerous publications have been conducted on this particular subject. In Iraq, Al-Bakray and Daoud (2005) recorded *E. Christensen* (36.5%) and *E. hirce* (18.5%) in goats at the Mosul abattoir. This aligns with findings by Diao *et al*. (2022), who discovered *Eimeria* species in goats in China, including *E. arloingi* (49.7%), *E. Christensen* (41.2%) and *E. hirce* (37.8%). Goz *et al*. (2006) identified *E. hirce* (18%) and *E. Christensen* (6%), were found in research on eimeriosis in goats done in Brazil by Cavalcante *et al*. (2012). This study is further comparable to molecular study by Bawn *et al*. (2020), who verified the COI sequences of *E. christenseni*, *E. hirci* and *E. arloingi* using COI and 18S r DNA sequences reporting sequence similarities of 98.9%, 98.4%, and 98.5% in Nay Pyi Taw area. Abdel Khader and Jarad (2022) in Al-Diwaniyah Province showed 87(87%) were positive for 18S rRNA gene of *Eimeria* spp. Seasonal differences the number of animals included in different researches, regional and agro-climatic diversity, local control approaches, and differences in the predominance of *Eimeria* species may all play a role in this variation.

CONCLUSIONS

The current study identified three species of *Eimeria* through morphological and molecular analyses by targeting the18S rRNA and COI genes (amplicons size H 454 bp and 405 bp). The identified species include: *Eimeria arloingi*, *E. hirci*, and *E. christenseni*. Hence, it is imperative to conduct a thorough research to mitigate the infections caused by *Eimeria* in goats. This necessitates the development of a comprehensive database to effectively reduce the prevalence of these parasites. Additionally, the use of anticoccidial medications and maintaining sanitary conditions are vital control measures that must be implemented. The current study identified three species of *Eimeria* according to morphological and molecular study by targeting 18S rRNA and COI gene (amplicons size H 454 bp and 405 bp) including: *Eimeria arloingi*, *E. hirci*, and *E. christenseni*. Hence, it is

imperative to conduct thorough research to mitigate the infection caused by *Eimeria* in goats; this necessitates the development of a comprehensive database to effectively reduce the prevalence of these parasites. Additionally, the utilization of anticoccidial medications and the maintenance of sanitary conditions are vital control measures that must be implemented. Furthermore, it is essential to regulate open habitat grazing and implement updated preventative measures, while also conducting further research on eimeriosis, especially in Waist Province, giving the limited studies on goats in this region and Iraq in general.

CONFLICT OF INTEREST STATEMENT

"Regarding the publishing of this article, the authors declare that they have no conflicts of" interest.

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عزل و تشخيص انواع جنس *Eimeria* Schneider, 1875 (Apicomplexa, Eimeriidae) من الماعز في محافظة واسط، العراق

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الخلاصة

يعد مرض الأيميريا من المشاكل الرئيسية التي تواجه المجترات في جميع أنحاء العالم. ويحدث هذا المرض في الغالب بسبب أنواع الأيميريا المتخصصة لكل مضيف والتي تنمو في الأمعاء الدقيقة والغليظة للحيوانات. إن الخسائر الناجمة من العدوى دون السريرية (فقدان الوزن على وجه الخصوص) والأمراض السريرية (الإسهال) تجعل أنواع هذا الجنس مصدر قلق اقتصادي كبير للغاية. لذلك، أجريت هذه الدراسة في بعض مناطق محافظة واسط. حيث تم جمع 180عينة من براز الماعز، من كلا الجنسين، شملت فئات عمرية وأشهر مختلفة، وتم فحص جميع عينات البراز بالمجهر وتم أخذ 75 عينة براز موجبة للفحص الجزيئي وتحليلها بشكل أكبر باستخدام تفاعل البوليميراز المتسلسل التقليدي والتسلسل والتحليل النشوئي. وأظهرت النتائج المجهرية أن المعدل الإجمالي للإصابة كان 41.6٪. حيث تراوحت معدل الإصابة بأنواع الأيميريا من 5.55٪ إلى 22.22٪ لتحديد ثلاثة أنواع مختلفة لجنس *Emeria* Schneider, 1875، وشملت الانواع الاتية :

E. arloingi Martin, 1909 (Marotel, 1905) (22.22٪) ، *E. christenseni* Levine, 1962 (88,13٪)، و *E. hirci* Chevalier, 1966 (5,55 ٪) فيما يتعلق بتفاعل PCR، أظهرت نتائج جين COI , 18 S rRNA والتسلسل الجيني أن عينات البراز كانت إيجابية لانواع الجنس *Eimeria* Schneider, 1875.