Research Article

Molecular Identification and Phylogenetic tree Analysis of Eimeria spp from Dogs in Al-Diwaniya Province, Iraq

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Abstract | Eimeria is a genus of parasites belonging to the family Eimeriidae which consisting of numerous species responsible for coccidiosis of various animals such as sheep, cattle, goats, swine, horses, cats and dogs. The protozoa are classified as enteric coccidia and monoxenous which is require a single host to complete their lifecycle. The primarily infects to the digestive tracts of carnivores, omnivores and herbivores which its leading to diarrheal condition known as coccidiosis. This study focused on assessing prevalence of Eimeria infections of dogs to obtain molecular identification of *Eimeria* species, to make phylogenetic tree analysis and to determine the risk factors related to infection. In Iraq's Al-Diwaniya province, 200 fecal samples were collected from dogs of various ages and sexes to conduct flotation tests and PCR analysis and phylogenetic tree examination. The overall prevalence of Eimeriosis of dogs in the present study was 13% based on PCR tech nique and 26% with flotation method. The native genotype in our study identified with four genotypes reported in the gene bank with the accession numbers MG825662. 1, MG825666. 1, LC380054. 1 and HM070377. 1. There were many risk factors related to high prevalence of Eimeriosis in dogs including: female dogs, 3 months until1 year age, stray dogs, ground house floor, time of house floor cleaning longer than a month, winter seasons and southern parts of the province. The higher prevalence of Eimeriosis infection is in stray dogs especially female dogs with 3 months to1 year of age.

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Introduction

Eimeriosis have been reported in a very wide range of birds and vertebrate animals including sheep, cattle, buffaloes, deer's, and many wild animals (Skirnisson and Cuyler, 2016, Fehlberg *et al*., 2018; Kanwal *et al*.,2022). The *Eimeria* species are commonly host specific. The *Eimeria* species are common gastrointestinal parasites which invades intestinal mucosa for many animal species including ruminants , wild animals and dogs (Sudan *et al*., 2012; Bangoura and Bardsley, 2020). In dogs the disease may be more serious when the animals are reared in overcrowded kennels especially in winter seasons when the floor of kennels are contaminated with the infective oocysts (Ryan *et al*., 2001; Abbas *et al*., 2023). This parasite

has exogenous and endogenous phases phases of life cycle, the exogenous phase occurs outside the animal bodies in the favorable hot and moist environments and the endogenous phase in which the parasite gets a very large number of divisions in the intestinal cells (Maheswari *et al*., 2023; Farooq, *et al*., 2023). The main root of infection is through the ingestion of contaminated water and food with the infective sporulated oocytes (Bowman, 2007; Zajac and Conboy 2012; Salad *et al*., 2025). The morbidity rate of this disease may approach 10-40% especially in the overcrowded kennels but the mortality rate may be very low (Mohamed and Al-Barwary, 2016). There are many *Eimeria* species in the world including *E. canis, E. cynomysis, E. parva, E. perforance, E. magna and E. stiedai* (Ryan *et al*., 2001; Skirnisson and Dyszynski, 2020). The clinical cases of infection are characterized by abdominal pain, diarrhea, tenesmus, weakness, anorexia and dehydration which may leads to the death of the infected animals (Charles *et al*., 2007; Ekawasti *et al*., 2021). The subclinical cases of infection are characterized by alterations in intestinal physiology, anorexia resulting in weight gain and poor growth of the young animals (Skirnisson and Dyszynski, 2020). The diagnosis of *Eimeria* species are performed by morphological identification through using taxonomic informations but it is difficult to differentiate between some species (Guardhouse and Eshar, 2014). Also there may be morphological changes of some species due to the use of some antiparasitic therapies prior of sampling time (Li *et al*., 2010; Jarad, 2020). The molecular techniques are used for identification of different *Eimeria* species which is a reliable, more sensitive and time efficient technique for identification of *Eimeria* infections in animals (Carvalho *et al*., 2011; Ekawasti *et al*., 2022). The PCR reports of *Eimeria* species in dogs are very limited worldwide (Hastutiek *et al*., 2022). So far as there is no molecular studies have been performed in Al-Diwaniya province, Iraq to detect the *Eimeria* species affecting dogs. The objectives of the present study were to investigate prevalence of *Eimeria* of dogs to obtain conduct molecular identification of *Eimeria* species and to perform phylogenetic tree analysis.

Materials and Methods

Fecal samples

In this current study, 200 fecal samples were collected from varying ages and sexes of dogs. The samples were obtained either directly from rectum using plastic gloves or from the ground immediately after defecation and described method by Guarnera *et al*. (2000). Each sample was placed in a labeled plastic container, stored in a cool box and transported to the research laboratory at the College of Veterinary Medicine for analysis.

Carpological examination

Fecal samples were analyzed microscopically by using the flotation concentration technique.

Flotation method

This method was performed for detecting Eimeria oocysts , 4-5gm of faeces were placed in beaker and mixed with tap water, then sieved and the fluid transferred to clean test tubes (15ml) then Centrifuged at 1000 rpm for 3 minutes, the supernatant was discarded. Sheather's solution was introduced to the sediment and thoroughly mixed before undergoing centrifugation again at the same speed for 5 minutes. Following this drop of solution was carefully extracted from the surface by using pipette, placed onto a slide, covered with a coverslip and examined under 10X and 40X magnifications (Dryden *et al*., 2005; Bibi *et al*., 2025).

Molecular study

A molecular study was carried out to detect Eimeria of dogs and to establish phylogenetic relationships. The research involved following steps:

DNA Extraction

The DNA were extracted from fecal samples by using AccuPrep® stool DNA Extraction Kit, Pioneer. Korea and performed according to company instructions.

DNA estimation

DNA extracted from fecal samples was analyzed by using Nano Drop spectrophotometer (Thermo, USA) to determines purity and concentration of DNA by measuring absorbance at 260/280 nm.

Polymerase chain reaction (PCR)

Polymerase chain reaction, commonly known as PCR which is a widely-used molecular biology technique designed to amplify and create multiple copies of specific DNA sequences. By utilizing series of temperature cycles and specialized enzymes like DNA polymerase. This technique has found extensive applications in various fields, including medical diagnostics, forensic science, genetic research,

and biotechnology PCR techniques were utilized to identify Eimeria spp. in DNA extracted from fecal samples. The procedures were implemented following the guidelines provided by the manufacturer (Table 1).

Table 1: *PCR Thermo cycler conditions were done by using conventional PCR thermo cycler system.*

PCR step	Temp.	Time	repeat
Initial Denaturation	95 °C	5min	1
Denaturation	95 °C	30sec.	30 cycle
Annealing	52 °C	30sec.	
Extension	72 °C	60sec.	
Final extension	72 °C	5min.	1
Hold	4 °C	-	-

Primers

The primers utilized in this study for the detection of *Eimeria* species were supplied by Pioneer Company., Korea as following (Table 2).

Table 2: *PCR primers internal transcribed spacer 1, partial sequence gene in Eimeria spp.*

Primers	Sequence		Amplicon
Eimeria ITS1	F	CTTCAGCT-TTCGACGGTAGG	494bp
	R	CTGCAGTATTCAA-GGCGACA	

Results

The fecal sample analysis using the flotation method yielded the following results. Out of a total, 200 fecal samples examined, 52 samples (26%) were infected with oocysts of Eimeria parasite, while 148 samples (74%) were free from oocytes of parasite (Table 3) and (Figure 1).

Table 3: *Overall percentage of eimeria sp. in dogs by flotation method and PCR technique.*

No. of dogs examined	Infected with Eimeria sp. microscopically		Non infected with Eimeria sp. microscopically	
200	positive	%	Negative	%
	52	26	148	74
	Infected with eimeria sp. by PCR		Non infected with eimeria sp. by PCR	
	positive	%	Negative	%
	26	13	174	87

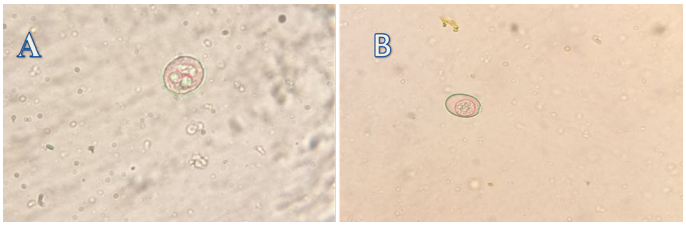


Figure 1: *Speculated oocyte of Eimeria , B : unsporulated oocyte of Eimeria.*

PCR technique

Eimeria spp. were detected in 26 (13%) of fecal samples from dogs by using PCR – technique, through using specific primers designed according to the gene of ITSI which was used for detection of *Eimeria spp.* protozoa at (494 bp) on agars gel electrophoresis, while 174 (87%) were free from the parasite, as shown in Figure 2.

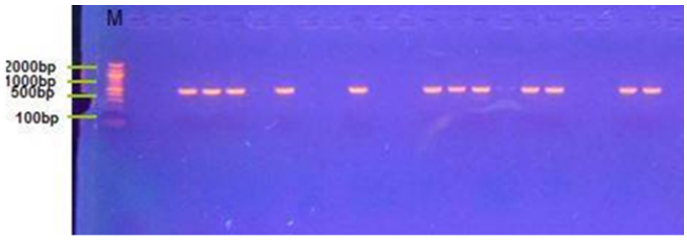


Figure 2: *Agarose gel electrophoresis shows the PCR product analysis of DNA extracted from fecal samples of the dogs, where Lane (M) ladder (100-2000 bp), other lane shown positive Eimeria spp. at (494bp) PCR product size*

DNA sequence and phylogeny tree

The local *Eimeria* isolates were uploaded to the NCBI Gene Bank database, where their accession numbers were retrieved as outlined in (Table 4). A phylogenetic tree was generated EGA version 6. 0 showing al Eimeria

Eimeria isolates to be closely related to the *Eimeria* sequences listed in the NCBI Blast database. The identities varied as illustrated in Table (4) and Figure 3 and Figure 4.

The results of sequencing of the nucleotides of the native genotype revealed presence of 4 genotypes in the world identifies with the native genotype in our study with higher percentage of identity 100% with the genotypes which were obtained from gene bank under the accession numbers MG825662. 1 and MG825666. 1 from Polland. Also the results of sequencing of the nucleotides of the native genotype of *Eimeria* species revealed identity percentage of 99.

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Figure 3: *Multiple sequence alignment analysis of small subunit ribosomal RNA gene in local Eimeria isolates and NCBI-Genbank Eimeria spp. isolates.*

Table 4: *The NCBI-BLAST Homology Sequence identity (%) between local Eimeria species of dog isolates and NCBI-BLAST submitted Eimeria species isolates*

Eimeria isolate	Genbank Accession number	NCBI-BLAST Homology Sequence identity (%)			
		Identical Eimeria	Number of Genbank Accession	Country	Identity (%)
No. 1	MK894303	Eimeria	MG825666. 1	Poland	100%
No. 2	MK894304	Eimeria	MG825662. 1	Poland	100%
No. 3	MK894305	Eimeria	LC380054. 1	Japan	99. 80%
No. 4	MK894306	Eimeria	HM070377. 1	USA	97. 17%

Table 5: *The relative risk of several factors related to the prevalence of Eimeria infection based on PCR- technique.*

Factors	No. of tested dogs	PCR – technique			
		No. of positive (%)	Relative risk (RR)	95% Confidence interval (CI)	P-value
Gender					
Male	96	7(7. 29) [a]	1		
Female	104	19(18. 26) [b]	2. 842	1. 137-7. 104	0. 021
Age					
< 3 months	60	2(3. 33) [a]	1		
1-5 years	67	6(8. 95) [a]	2. 852	0. 553-14. 708	0. 192
3 months-1 year	73	18(24. 65) [b]	9. 490	2. 103-42. 823	0. 0006
Type of dogs					
Pet dogs	56	3(5. 35) [a]	1		
Police dogs	62	8(12. 90) [a]	2. 617	0. 658-10. 403	0. 159
Stray dogs	82	15(18. 29) [b]	3. 955	1. 087-14. 381	0. 026
House floor					
Concrete	106	8(7. 54) [a]	1		
Ground	94	18(19. 14) [b]	2. 901	1. 197-7. 030	0. 014
House floor cleaning					
Once amonth	102	7(6. 86) [a]	1		
Longer than a month	98	19(19. 38) [b]	3. 264	1. 305-8. 162	0. 008

80% with the genotype reported in the gene bank under the accession number LC380054. 1 from Japan. Moreover the results of sequencing of the nucleotides of the native gene of *Eimeria* species revealed identity percentage of 97. 17% with the genotype reported in the gene bank under the accession number HM070377. 1 from United States of America. According to the results of PCR – technique the prevalence of *Eimeriosis* in the present work was significantly higher in female dogs(bitchs) 18. 26% [Relative risk (RR): 2. 842 times , Confidence interval(CI): 1. 137-7. 104] than the male dogs 7. 29%. The prevalence of *Eimeriosis* in the present study was significantly higher in 3 month-1year old dogs

Table 6: *The relative risk of seasonal factors related to the prevalence of Eimeria infection based on PCR technique.*

Seasonal factors	No. of tested dogs	PCR – technique			
		No. of positive (%)	Relative Risk (RR)	95% Confidence Interval	P- value
Summer	54	2(3. 70) [a]	1		
Autumn	48	4(8. 33) [a]	2. 363	0. 413-13. 523	0. 321
Spring	46	6(13. 04) [a]	3. 900	0. 747-20. 359	0. 084
Winter	52	14(26. 92) [b]	9. 578	2. 054-44. 661	0. 0008

Table 7: *The relative risk of geographical factors related to the prevalence of Eimeria infection based on PCR technique.*

Geographical factors	No of tested dogs	PCR – technique			
		No of positive (%)	Relative risk (RR)	95% Confidence Interval (CI)	P- value
Central parts	36	1(2. 77) [a]	1		
Eastern parts	35	2(5. 71) [a]	2. 121	0. 183-24. 511	0. 538
Northern parts	38	4(10. 52) [a]	4. 117	0. 437-38. 740	0. 184
Western parts	42	5(11. 90) [a]	4. 729	0. 526-42. 526	0. 131
Southern parts	49	14(28. 57) [b]	14. 000	1. 745-112. 308	0. 002

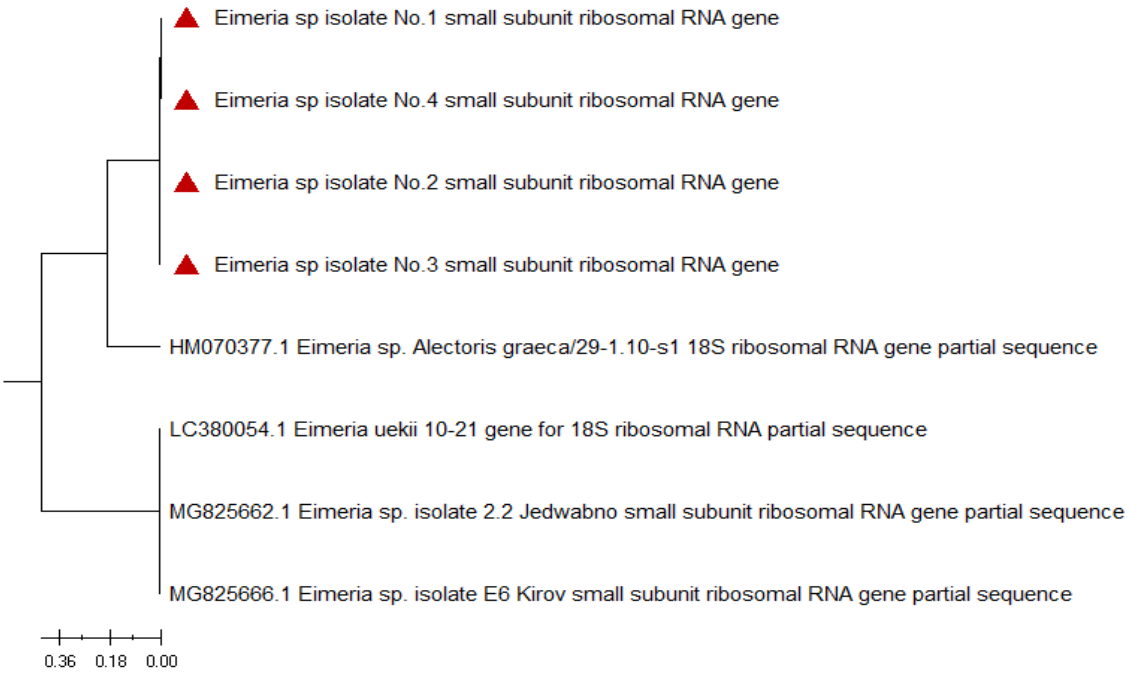


Figure 4: *Phylogenetic tree analysis based on small subunit ribosomal RNA gene partial sequence in local Eimeria dogs isolates that used for genetic Eimeria species identification.*

24. 65% (RR: 9. 490 times, CI: 2. 103-42. 823) than the 1-5 years and < 3 months old dogs 8. 95% and 3. 33% respectively. The prevalence of *Eimeriosos* in the current study was significantly higher in in stray dogs 18. 29% (RR: 3. 955 times , CI: 1, 087-14. 381) than police and pet dogs 12. 90% and 5. 35% respectively. In the current work the prevalence of *Eimeriosis* was greater in ground house floor dogs 19. 14% (RR: 2. 901 times, CI: 1. 197-7. 030) than the concrete house floor dogs 7. 54%. The prevalence of *Eimeriosis* was significantly higher in longer than a month period of house floor cleaning 19. 38% (RR: 3. 264 times, CI: 1. 305-8. 162) than once a month of house floor cleaning 6. 86% (Table 5). The present study revealed a higher significantly prevalence of Eimeriosis through winter season which was 26. 92% (RR: 9. 578 times, CI: 2. 054-44. 661) than the summer, autumn and spring seasons which were 3. 70%, 8. 33%, 13. 04% respectively (Table 6). The prevalence of *Eimeriosis* in the current study was significantly higher in the

southern parts of Al-Diwaniya province 28. 57% (RR: 14. 000 times, CI: 1. 745-112. 308) than the central, eastern, northern and western parts of the province which were 2. 77%, 5. 71%, 10. 52%, 11. 90% respectively (Table 7).

Discussion

Eimeria parasites are intracytoplasmic parasites which invade and destroy the intestinal epithelial cells of the infected dogs leading to digestive dysfunction, chronic or acute damage to the intestinal mucosa of the infected animals (Saratsis *et al*., 2012; Hllytchaikra *et al*., 2018). The clinical symptoms of the infected animals, morphology of oocysts and the typical post mortem lesions are very important for identification of *Eimeria* species in dogs (Gardhouse and Eshar, 2014), but the PCR – technique is the gold standard approach for detection of *Eimeria* species in the infected animals (Li *et al*., 2010; Mehdi *et al*., 2018). This study based on the findings of the PCR technique the prevalence of Eimeriosis in dogs was determined to be 13%. This is notably higher compared to the results reported by Kohansal *et al*. (2017) who indicated a prevalence rate of 0. 7% of dogs in Iran by using the sedimentation concentration method and modified Ziehl-Neelsen staining. The present study indicates a higher prevalence of Eimeriosis in dogs compared to the findings of Maheswari *et al*. (2023) who documented a prevalence rate of 1. 33% among Kintamani Bali dogs in the Kintamani district. Indonesia through using the floating method. The prevalence of *Eimeriosis* in the current study was higher than the results of Mohamed and Al-barwary, (2016) who reported that the prevalence of *Eimeriosis* in dogs was 3. 7% in Duhok province, Kurdistan region, Iraq by using flotation technique and sedimentation technique. In the current study the prevalence of *Eimeriosis* in dogs was lower than the results of Beiromvand *et al*. (2013) who mentioned that the prevalence of *Eimeriosis* in dogs was 19% in the Chenaran County, Razavi Khorasan Province, Iran through using sieving/flotation and modified Ziel-Neelsen staining technique. This variations in the prevalence may be due to various factors, including environmental variations, the climate, age of the animals, husbandry procedures and management systems (Rehman *et al*., 2011). In the present work the prevalence of *Eimeriosis* was higher in the female dogs (batches) more than the male dogs and this finding is consistent with the results of Rehman *et al*. (2011); Mohamaden *et al*. (2018) who mentioned that the prevalence of *Eimeriosis* was higher in the female animals than the males because the female animals are exposed to many physiological stresses which is related to pregnancy, parturition and lactation which makes them more susceptible to the infections with *Eimeria* species more than the males. The prevalence of Eimeriosis in the current study was higher in 3 month-1 year old dogs than the adult dogs with 1-5 years old and the young puppies with < 3 months age, and this result is compatible with the results of (Lopez-Osorio *et al*., 2020) who reported that the prevalence of *Eimeriosis* is higher in the growing animals more than adult animals , and this may be attributed to the higher protective immunity in the adult animals than the growing animals and the young animals < 3 months have a higher maternal immunity due to the high levels of the antibodies in the colostrum. In the present study the prevalence of *Eimeriosis* in the stray dogs was higher than the pet and police dogs which may be attributed to the fact that the stray dogs consumes the rabbit , rodent, cat, chichen, wild animal and other domestic animal farces in their area and this coprophagic behavior in the stray dogs were observed publicly and plays important role in the transmission of *Eimeria* from other animal species to the dogs (Frenkel and Parker 1996; Al-Mousawi et. al., 2024) while the pet and Police dogs have specific feeding programs in their owner house and kennels and don't have coprophagic behaviors like the stray dogs. In the current work the prevalence of *Eimeriosis* was higher in ground house floors than the concrete house floors and this result is compatible with the results of Kohansal *et al*. (2017), Keomoungkhoun *et al*. (2023) who mentioned that the animals reared on the ground house floor had a greater infections with Eimeria species than the animals reared on the concrete house floor because the cleaning of the concrete floor is easier than the ground floor. In the present work the prevalence of *Eimeriosis* was higher when the house floor cleaning was longer than a month than once amonth cleaning of the house floor, and this observation is compatible with the findings of and Keomoungkhoun *et al*. (2023) who reported that the prevalence of Eimeriosis was higher in the longer than a month of the house floor cleaning period than the once a month cleaning period due to the more contamination of the floors with the oocytes of *Eimeria* species. The prevalence of *Eimeriosis* in dogs in the present study was higher in the winter season than the summer, autumn and

spring seasons and this result is consistent with results of El-Seify *et al.* (2012) and Fadly, (2012) who reported that the prevalence of *Eimeriosis* was higher in the winter seasons than other seasons which may be due to presence of high levels of rains during the winter seasons that increases the distribution and contamination of the ground with the infective oocysts. These oocysts and the *Eimeria* parasites can remain viable and infectious for many years in vitro leading to many adverse environmental effects due to a thick oocyte wall. In the current study the prevalence of *Eimeriosis* was higher in the southern parts of Al-Diwaniya Province than the central, eastern, northern and western parts of the province, and this result is compatible with results of Ren *et al.* (2021) who reported that the prevalence of *Eimeriosis* in Sichuan region which is located in the southwest parts of China was higher than Shaanxi which is located in the central parts of China which me be attributed to differences in soil conditions, rainfall environments, vegetation, humidity, temperature and other factors due to differences in the climate may affect on the distribution of the oocytes and *Eimeria* parasites. On the contrary (Malek and Kuraa, 2018) reported that the prevalence of *Eimeriosis* was higher in summer seasons than winter, autumn and spring seasons. The results of sequencing of the nucleotides of the native genotype revealed presence of 4 genotypes in the world identifies with nucleotides the native genotype in our study with higher percentage of identity 100% with the genotypes which were obtained from gene bank under the accession numbers MG825662. 1 and MG825666. 1 from Poland. Also the results of sequencing of the nucleotides of the native genotype of *Eimeria* species revealed identity percentage of 99. 80% with the genotype reported in the gene bank under the accession number LC380054. 1 from Japan. Moreover the results of sequencing of the nucleotides of the native genotype of *Eimeria* species revealed identity percentage of 97. 17% with the genotype reported in the gene bank under the accession number HM070377. 1 from United States of America. The clustering observed within the same nodule suggests a close relationship among involved entities, likely influenced by the geographic proximity of the countries in question. This proximity may follow a shared migratory path for paratenic hosts such as rodents and birds across different nations along a common geographic line. The findings of this study incorporate data from multiple investigations that have documented genetic variation and phylogenetic relationships based on nuclear gene sequences. The sequences have been utilized to assess genetic diversity in *Eimeria* and other protozoa parasites worldwide. The results revealed low genetic variation in the nuclear ribosomal ITS-1 region of *Eimeria* isolates from Iraq compared to those found in other countries. It was concluded that DNA sequencing combined with phylogenetic analysis serves as a valuable tool for understanding the evolutionary relationships among organisms.

Conclusions and Recommendations

The current study revealed that Eimeriosis is evident in dogs in Al-Diwaniya province, Iraq and there are four genotypes in the world identifies with the native genotype under the accession numbers MG825662. 1, MG825666. 1, LC380054. 1and MH070377. 1.

There were several risk factors related to high prevalence of Eimeriosis including: Female dogs, 3 months-1 year ages, stray dogs, ground house floor, longer than a month house floor cleaning, winter season and southern parts of the province.

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Novelty Statement

The originality of this study lies in the fact that it provides first comprehensive molecular and genetic analysis of Eimeria strains in dogs of Diwaniyah Governorate in Iraq which linking them to global genetic patterns and identifying precise environmental factors (such as cleaning habits and type of flooring) that contribute to spread of infection.

Author's Contribution

Noor I. Jarad and Mozhir K. K. Almahdawi: The authors mentioned in this manuscript contributed to preparation of the study plan, fieldwork, laboratory analysis, data collection, statistical analysis, and interpretation of results. Supervised for follow-up of the experimental research work and wrote draft of the

manuscript.

Alyaa S. K. Al-Shammari , Abdul Sattar S. S. Al-Bayati: The authors contributed to the following tasks: laboratory analysis of samples, manuscript review, and correction of spelling and grammatical errors. They prepared the raw materials required for this study, wrote the research draft, and reviewing of the manuscript references.

Generative AI and AI-assisted technology statement

The authors declare that no generative AI and AI assisted technology was used in the creation of this manuscript.

Conflict of interest

The authors have declared no conflict of interest.

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