

**Special Issue:**

Emerging and Re-emerging Animal Health Challenges in Low and Middle-Income Countries

Investigation of *Cryptosporidium* Species Infecting Dogs

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Abstract | The percentage of dog breeding has increased recently, however, no epidemiological or molecular data regarding *Cryptosporidium* species infection in dogs is available in Iraq. Therefore, the current study aimed to detect and identify *Cryptosporidium* species infecting dogs using microscopy and molecular techniques. For the microscopic examination, feces samples of 100 dogs were examined from September 2023 to March 2024 by the flotation technique based on the modified Ziehl–Neelsen-staining technique. The results showed that the overall percentage of *Cryptosporidium* infection was 22% (22 out of 100). These results also found that the infected males recorded the highest infection rate compared with infected females, where the percentages were 22.41% (13/58) and 21.42% (9/42), respectively. In addition, the infection rate of infected animals less than one year was higher than those aged more than one year, with percentages of 23.18% (16/69) and 19.35% (6/31), respectively. The findings also demonstrated that the infection rate was high in infected dogs in rural areas 23.33% (14/60), in comparison with those living in urban areas 20% (8/40). Regarding the molecular techniques, two species (*C. Canis*, and *C. parvum*) were genetically identified based on the PCR and DNA sequencing techniques using the gene: 60 kDa glycoprotein (gp60) gene sequence. We conclude the first molecular study in Babylon province that detected and identified these two species in dogs. Interestingly, the species of *C. parvum* is a zoonotic species that can transmit the infection to humans, highlighting its epidemiological importance of *Cryptosporidium*.

Keywords | *Cryptosporidium*, Dog, Zoonotic species, Nested PCR, DNA sequencing**Received** | June 15, 2024; **Accepted** | October 01, 2024; **Published** | December 04, 2024***Correspondence** | Suhad I.J. Al-Asady, Department of Public Health, Babil Health Directorate, Ministry of Health, Iraq; **Email:** suhad-alasady@vet.uoqasim.edu.iq**Citation** | Al-Asady SIJ, Al-Hasnawy MH (2024). Investigation of *Cryptosporidium* species infecting dogs. J. Anim. Health Prod. 12(s1): 174-181.**DOI** | <https://dx.doi.org/10.17582/journal.jahp/2024/12.s1.174.181>**ISSN (Online)** | 2308-2801**Copyright:** 2024 by the authors. Licensee ResearchersLinks Ltd, England, UK.This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

INTRODUCTION

Cryptosporidium species is a common protozoan that affects domestic and wild dogs (Taghipour *et al.*, 2020). Animals with symptomatic or asymptomatic cryptosporidiosis poses a risk infection to humans and other animals, where the reinfection is common unless infectious abscesses are eliminated in environment (Nichols, 1992; Khan *et al.*, 2023). Cryptosporidiosis typically manifests in acute to severe diarrhea along with abdominal pain. In immunocompetent individuals, the course of infection is

normally self-limiting. Dogs rarely show signs of infection and act as asymptomatic carriers (Thompson *et al.*, 2016). Considering the close contact between companion animals and their owners, the role of dogs as a potential risk factor for the infection with zoonotic pathogens comes to the center of attention (Murnik *et al.*, 2022). Various animal species that serve as reservoirs host parasites harboring *C. parvum* capable of infecting humans. The extent of the parasite's danger to public health (Ali and Ali, 2023; Jarad, 2020; Al-Musawi *et al.*, 2022; Alali *et al.*, 2021), *Cryptosporidium* parasite can be diagnosed in the laboratory through a fecal

microscopic examination, various immunological tests, and molecular methods. Research using nested PCR (nPCR) has been shown to be more effective, accurate and useful for diagnosing *Cryptosporidium* species infections compared to the microscopic examination (Smith, 2007). While there are other methods available to detect *Cryptosporidium* oocysts in feces, such as microscopic staining and surface antigen detection, these techniques are not enough to identify the species and genotypes due to the identical appearance and antigens of the oocysts (Chalmers and Katzer, 2013; Tamura et al., 2021). Molecular methods like conventional PCR, Nested-PCR, and sequencing identify and characterize the parasite at species and subspecies levels using *gp60* genes (Al-Hassan and Al-Safo, 2024; Kadim et al., 2022; Faraj, 2019).

Cryptosporidium species are important on public health and has economic implications in the country due to the risk of the outbreak of the disease and its associated conditions of economic losses. It was mentioned that prevalence of *Cryptosporidium* oocysts was recorded by some studies in Iraq depending on microscopic and molecular techniques; however, the investigation of this infection in other parts of the country is still incompletely done (Alali et al., 2021). Previous studies performed in Iraq showed the presence of *Cryptosporidium* species in dog in Baghdad by Faraj (2019), in Karbala Province Iraq by Al-Yasary and Faraj (2021), Mosul city, Iraq by Hadi et al. (2014). However, there is no previous epidemiological or molecular data regarding *Cryptosporidium* spp. Infection in dogs in Babylon province. Thus, this study aimed to detect and identify *Cryptosporidium* species in dogs in this region using microscopic and molecular techniques.

METERIALS AND METHODS

SAMPLES COLLECTIONS, PERIOD AND LOCATION OF THE STUDY

This study was conducted in Babylon province from September, 2023 to March, 2024 using the microscopic and molecular techniques for diagnosing *Cryptosporidium* species in dogs. For the microscopic examination, fecal samples (approximately 10 g) of 100 dogs were collected in sterile plastic cups labeled with necessary data, such as

sex, age, and date. These samples were also collected from different locations (zoos, veterinary clinics, and houses), then transferred with icebox directly to the advance parasitic laboratory.

FECAL SAMPLES EXAMINATION

Each fecal sample was divided into 2 parts: The first part was checked using the Sheather's sugar flotation technique based on the modified Ziehl–Neelsen-staining technique, where it was examined microscopically under 40 ×, 100x magnifications for the detection of *Cryptosporidium* oocysts according to parasitology keys (Henriksen and Pohlenz, 1981; Hassan et al., 2021) the second part was subjected to the DNA extraction and PCR analyses. *Cryptosporidium*-positive samples were also stored in 2.5% potassium dichromate at 4°C (Thompson et al., 2005) the dimensions of the parasite stages were measured using an ocular micrometer using a Leica microscope equipped with a digital camera (Scope Image 9.0, China). The camera software was calibrated for all microscope lenses using a 0.01 mm stage micrometer (ESM-11/Japan). *C. parvum* 4.5 × 5.0, *C. canis* 4.7 × 5.0 infected dog (Mohamed, 2014).

MOLECULAR STUDY

The current study utilized the Nested-PCR technique to molecularly detect *Cryptosporidium* spp. in dog feces by targeting the GP60 genes. Later on, the sequencing method was employed to analyze and describe the different species of *Cryptosporidium*.

DNA EXTRACTION

Following the manufacturer's instructions, DNA was extracted directly from 50 fecal samples from dogs, including microscopically positive samples for confirmation and further identifications using (EasyPure®Stool Genomic DNA Kit, Transgen Company, China).

PRIMERS USED IN THE STUDY

NESTED PCR

Four sets of primers were used in this study according to Tables 1 and 2 for identifying two species of *Cryptosporidium*, where two sets of primers were for identifying (*C. parvum*) according to Table 1 and two sets of primers were for identifying (*C. canis*) according to Table 2.

Table 1: The sequence of primer of nested PCR that used for detecting *C. parvum*.

Primer	Sequence	Primer sequence 5'→ 3'	Tm (°C)	GC%	Size of product (bp)	Reference
First reaction <i>GP60 Cryptosporidium</i> spp.						(4)
First reaction GP60	F	ATAGTCTCCGCTGTATTC	51.7	44	921bp	
	R	GGAAGGAACGATGTATCT	53.1	44		
Second reaction <i>GP60 Cryptosporidium parvum</i>						
Second reac- tion GP60	F	TCCGCTGTATTCTCAGCC	59.1	56	887bp	
	R	GCAGAGGAACCAGCATC	58.4	59		

Table 2: The sequence of primer of PCR that used for detecting *C. canis*.

Primer	Sequence	Primer sequence 5'→3'	Tm (°C)	GC%	Size of product (bp)	Reference
GP60 <i>Cryptosporidium canis</i>						
Reaction GP60 F		AAACGCCTCACTCGTTTCCT	61.3	50	390 bp	ID: ON863571.1
R		AGTTCGTCCTTGCGAATCCA			Primer design	

PHYLOGENETIC TREE

To compute evolutionary distances, a phylogenetic tree was constructed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method (Moss and Hendrickson Jr, 1973; Tamura *et al.*, 2004). The optimal tree with the sum of branch length = 0.00795808 is shown of *C. parvum*. the optimal tree with the sum of branch length = 11.90711807 is shown of *Cryptosporidium canis* (Tamura *et al.*, 2013).

DNA SEQUENCING

DNA sequencing was conducted to identify *Cryptosporidium* species, and four nPCR-positive local isolate products were sent to Macrogen Company in Korea to identify *Cryptosporidium* spp. A homology search was conducted using the Basic Local Alignment Search Tool available at the National Center for Biotechnology Information (NCBI, <http://www.ncbi.nlm.nih.gov>) and the BioEdit program, version 7.2. The results were compared with data obtained from the GenBank and the ExpASY program (SIB Swiss Institute of Bioinformatics, Switzerland) available on the NCBI website (Mphephu *et al.*, 2021).

STATISTICAL ANALYSIS

The data was tabulated in a datasheet of IBM SPSS version 26.0, which was utilized to do the statistical analysis. The significant differences were tested using the analysis of the person chi-square test. Statistical significance was defined as a probability value ($p \leq 0.05$) (Firdaus, 2021).

RESULTS AND DISCUSSION

MICROSCOPIC EXAMINATION

The microscopy is one of the most widely used methods for the detection of *Cryptosporidium* oocysts in fecal samples in dogs. The observations appeared that 22 out of 100 (22%) fecal samples were found to be positive for the diarrhea causing protozoan (*Cryptosporidium* species). The microscopic examination results demonstrated that *Cryptosporidium* spp. oocyst appeared as a pink to red round bodies against blue background when stained with the modified ziehl-neelsen acid fast staining. The average size of these oocysts were ($\pm 4\mu\text{m} \times \pm 5.2\mu\text{m}$) as shown in the Figure 1.

According to sex, the infection rate in dogs was 13/85 (22.41%) in males while 9/42 (21.42 %) in female as shown

in the Figure 2, The highest infection rates were observed in animals under one year of age, with 16/69 (23.18 %) and above one year was 6/31 (19.35) as illustrated in the Figure 3. Rural areas had a higher rate of infection in dogs, where it was 14/60 (23.33 %) compared with urban areas 8/40 (20 %) Figure 4.

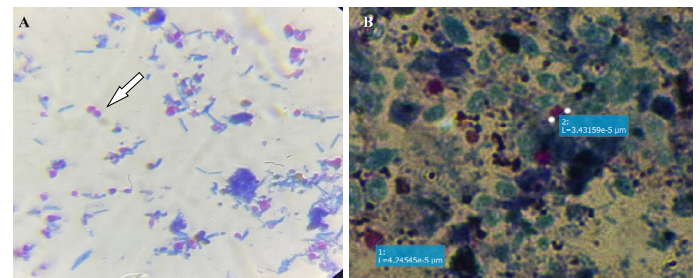


Figure 1: Morphology of *Cryptosporidium* spp.: A: Oocysts (40X) by using modified Ziehl-Neelsen stain. B: Oocysts (100X) by using modified Ziehl-Neelsen stain with oil immersion and using a digital camera

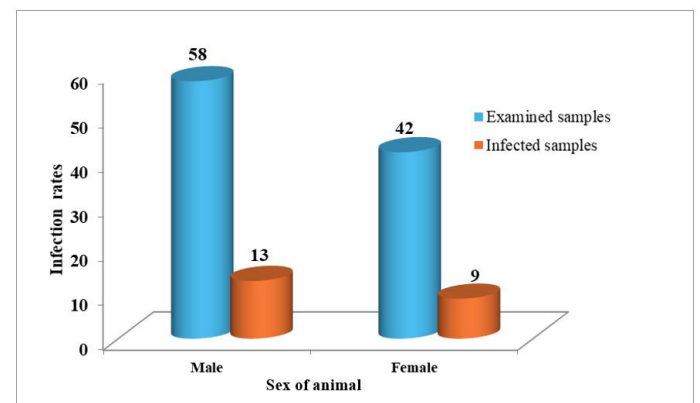


Figure 2: Infection rate of *Cryptosporidium* spp. in dog according to the sex.

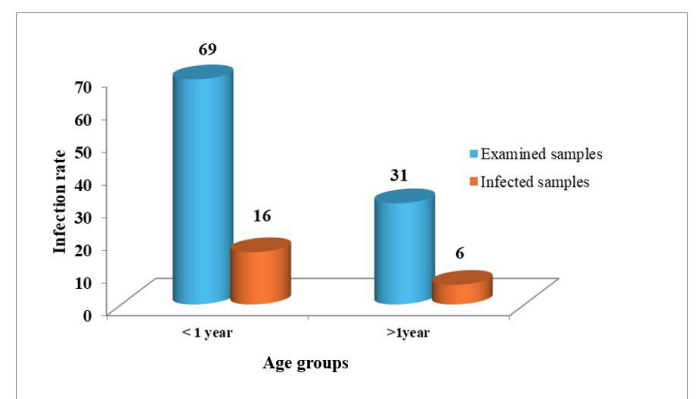


Figure 3: Infection rate of *Cryptosporidium* spp. in dog according to the age group.

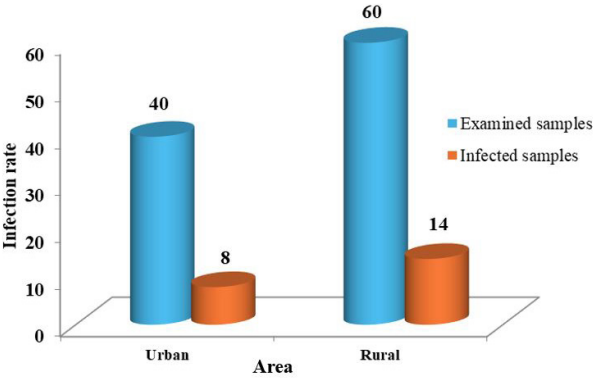


Figure 4: Infection rate of *Cryptosporidium* spp. in dog according to the area of study.

MOLECULAR DIAGNOSIS

The molecular diagnosis results showed that the nested PCR amplification of the GP60 gene was positive for 24 % (12/50) fecal samples of dogs 10% (5/50) of *Cryptosporidium canis* and 14% (7/50) of *Cryptosporidium parvum* (Figures 5, 6, 7).



Figure 5: Agarose gel electrophoresis image shows nested PCR product for *Cryptosporidium Canis*, GP60 gene 390bp in dogs' samples. Bands were fractionated by electrophoresis on a 1.5% agarose gel, and visualized under U.V. light after staining with red stain. Lane: M (M: 100bp-1500bp ladder). (M=marker) (CP= control positive) (CN= control negative).

DNA SEQUENCE ANALYSIS OF *CRYPTOSPORIDIUM* SPP.

To investigate *C. canis* and *C. parvum* in dogs in Babylon province, four isolates were genetically characterized using

GP60 gene sequencing. In dogs, sequencing revealed specific *C. canis* (1/4, 20 %) and *C. parvum* (3/4, 60 %), zoonotic species. *cryptosporidium* spp. isolates were submitted to the NCBI GenBank database, and GenBank accession numbers were obtained for 4 *Cryptosporidium canis* (1) and *Cryptosporidium parvum* (3) isolates: *C. parvum* that have been deposited in the gene bank under the accession numbers (PP707752.1; PP707753.1; PP707754.1). and identified *C. canis* gene (GP60) that have been deposited in the gene bank under the accession numbers (PP707750.1) as shown in the (Table 3 and Figures 8, 9, 10, 11).

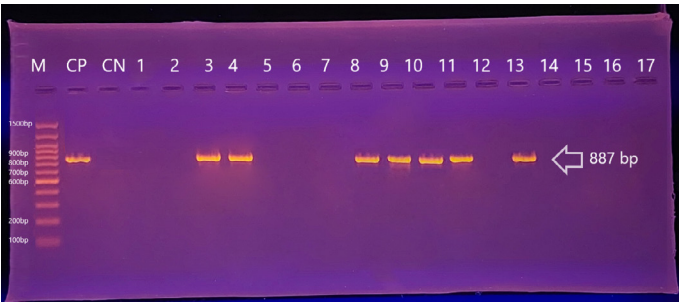


Figure 6: Agarose gel electrophoresis image shows nested PCR product for *cryptosporidium parvum*, GP60 gene 887bp in dogs samples. Bands were fractionated by electrophoresis on a 1.5% agarose gel, and visualized under U.V. light after staining with red stain. Lane: M (M: 100bp-1500bp ladder). M= marker, CP= control positive, CN= control negative.

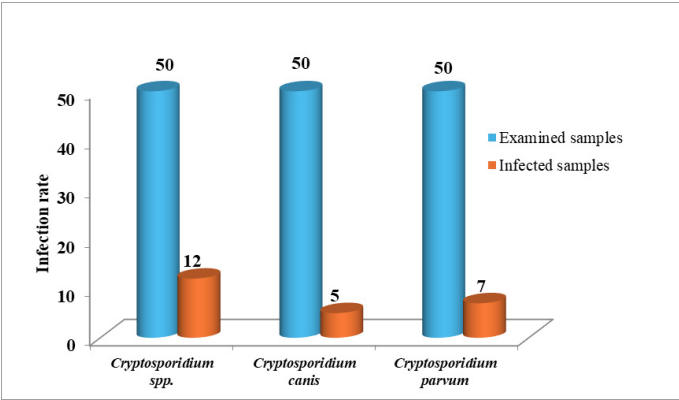


Figure 7: Infections rates of *Cryptosporidium* species based on the nested PCR.

Table 3: DNA sequence analysis of *Cryptosporidium* spp. based on 60 kDa glycoprotein (GP60) gene.

Gene : 60 kDa glycoprotein (GP60) gene							
Identities	Sequence ID with compare	Host	Source	Nucleotide	Location	Type of substitution	No. of sample
99%	ID: MT954608.1	Dog	<i>Cryptosporidium canis</i>	A\G	316	Transition	1
				T\G	384	Transversion	
100%	ID: GU214366.1	Dog	<i>Cryptosporidium parvum</i>	--	--	--	2
99%	ID: GU214366.1	Dog	<i>Cryptosporidium parvum</i>	T\C	331	Transversion	3
99%	ID: GU214366.1	Dog	<i>Cryptosporidium parvum</i>	T\C	331	Transition	4

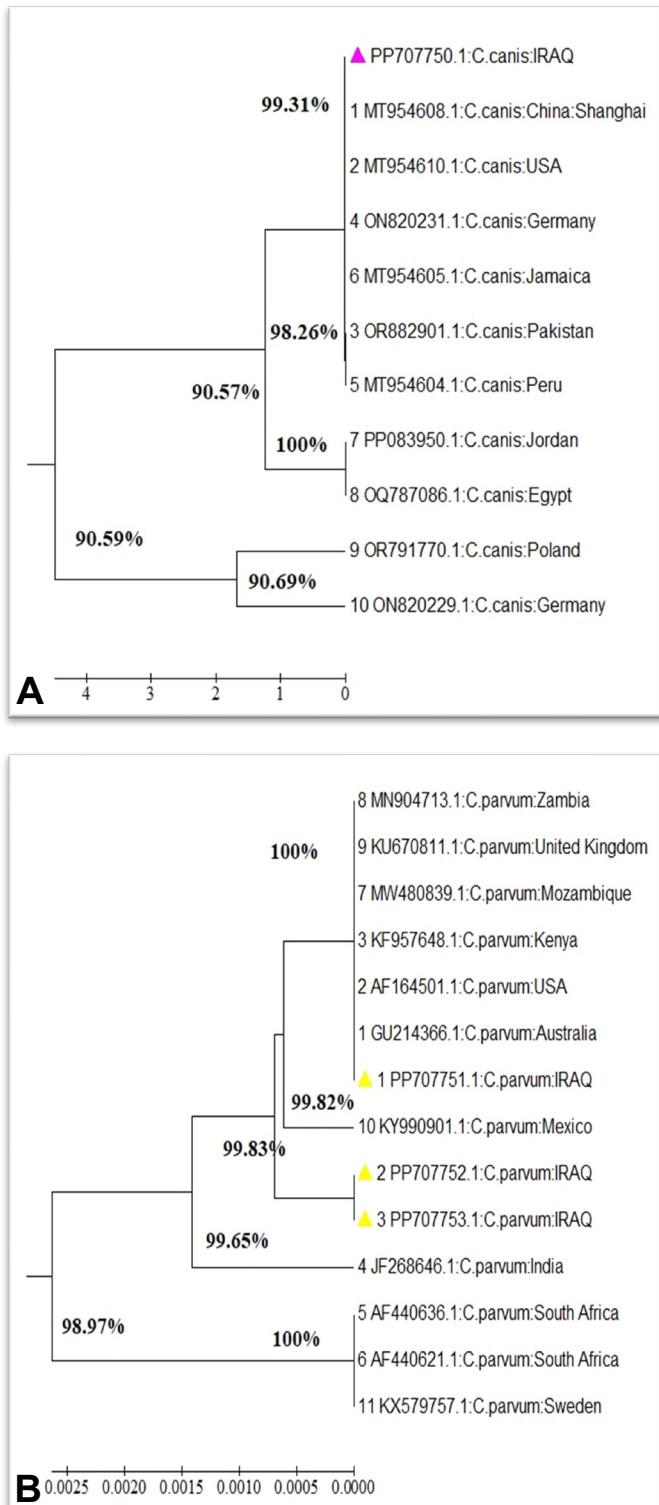


Figure 8: Phylogenetic tree analysis based on the partial sequence of GP60 gene explains identity between the local isolates of *Cryptosporidium canis* (A) and *Cryptosporidium parvum* (B) in dogs and NCBI-BLAST isolates.

PHYLOGENETIC TREE

PHYLOGENETIC TREE *CRYPTOSPORIDIUM CANIS*

Phylogenetic tree *Cryptosporidium parvum*: The evolutionary history was inferred using the UPGMA method (Moss and Hendrickson Jr, 1973). the optimal tree with the sum of branch length = 11.90711807 is shown.

the tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the maximum composite likelihood method (Tamura *et al.*, 2004) and are in the units of the number of base substitutions per site. the analysis involved 11 nucleotide sequences codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 281 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura *et al.*, 2013) as shown in the the (Figure 8A, B).

CONCLUSIONS AND RECOMMENDATIONS

According to the overall finding of the current study, it was shown that the results of the molecular techniques represented the first contribution to the knowledge of *cryptosporidium* species in dogs in Babylon Province/Iraq. Two species of the protozoan *Cryptosporidium* (specific species *C. canis* and zoonotic species *C. parvum*) have been successfully identified in dogs living in this area. Understanding the parasite species isolated in a certain area is crucial for providing accurate information about the risk of zoonotic transmission, host specificity, and the sources of infection during outbreaks. This analysis could also help define the role of *Cryptosporidium* spp. as an animal pathogen that harms animal health (Robertson *et al.*, 2020).

Through microscopic examinations, the infection rate of *cryptosporidium* spp. observed in dogs was 22%. This result was significantly agreed with a previous study done by Moreira *et al.* (2018). In Brazil was reported 24.63%. It was in agreement with (Gharieb *et al.*, 2018) in Egypt who found the rate of infection by using microscopic examination was 35%. In Iraq a study conducted by Al-Yasary and Faraj (2021) that reported 34.5 % in Karbala province. While it was not similar to the findings of (Hadi *et al.*, 2014) in Mosul city, Iraq, which recorded 42%. The current study did not also agree with other studies; (Lindsay and Zajac, 2004) in the USA were recorded the rates of infection 44.8% and (El-Madawy *et al.*, 2010) in Egypt 50%. The reason for the difference infection rates of *Cryptosporidium* spp. in dogs might be ascribed into the areas of study, numbers of examined animals, and techniques. In addition, the transmission occurs through direct contact between healthy and diseased dogs, as well as the availability of a favorable environment for oocysts to persist for extended durations (Arsalan *et al.*, 2006).

The study found no significant difference in the infection rate between females and males. However, it was found

that male dogs had a higher prevalence of *Cryptosporidium* spp. infection compared to female dogs, this result was consistent with that study in Egypt distribution of *Cryptosporidium* infection was higher in male household dogs than females 38.9% and 21.4%, respectively (Jian *et al.*, 2014). While it disagreed with a study of (Al-Yasary and Faraj, 2021) in Karbala province in Iraq which found the higher infection rate in female dogs 48.27% compared to male dogs 23.89%. current and previous research indicates that *Cryptosporidium* is quite prevalent, and both males and females of dogs have an equal probability of getting infected when exposed to contaminated substances (Olabanji *et al.*, 2016).

With regard to the effects of age on cryptosporidiosis, the results of present study found highest rate of infection in dogs with less than one year old compared to other age groups. The results were in agreement with the studies done in Iraq (Al-Yasary and Faraj, 2021; Faraj, 2019). Al-Yasary and Faraj (2021) who found that the infection rate was higher 55.69% and 20%, respectively in young's dogs than adults were 20.66% and 10%, respectively. The results were also consistent with research conducted in Egypt, which found a 50% infection rate in young pups compared to a 10% rate in adult animals (Gharieb *et al.*, 2018). The findings of the present study contradict a previous study conducted in Iran by Tavalla *et al.* (2017), which reported a higher infection incidence in adults at 12% and a lower infection rate in young individuals at 2.36% (Tavalla *et al.*, 2017). there is a strong link between both age and the danger of *Cryptosporidium* infection, which was explained by This finding might be attributed to immature immune systems of pope dogs, whereas these dogs could be more venerable to *Cryptosporidium* infection (Kvác *et al.*, 2006).

The results of the study showed non-significant differences between area of the study that include (rural and urban area). The higher infection rate was in rural area and the lower infection rate was in the urban area. Similar trends have been observed in studies in Egypt, 40% and 10% respectively (Gharieb *et al.*, 2018). Also a study of Al-Yasary in Karbala province in Iraq 47.5% in rural than 20% urban (Al-Yasary and Faraj, 2021). The elevated rates of infection in rural areas can be attributed to various factors, such as inadequate access to potable water, reliance on river water as a primary water source, exposure to contaminated soils in agricultural areas containing parasite cysts, frequent contact with animals acting as reservoirs for the parasite, utilization of animal waste as organic fertilizer, and lower levels of health and cultural development among rural populations (Mugala *et al.*, 2018).

According to the results of nested PCR and DNA sequencing techniques, the frequency of infection rates in DNA samples showed that samples were positive. In Iraq,

previous studies found the prevalence of *Cryptosporidium* spp. in dogs using nested PCR technique was 28.6% (Faraj, 2019; Al-Yasary and Faraj, 2021) Karbala province in Iraq, 47% by Faraj in Bagdad Province Iraq (Al-Yasary and Faraj, 2021). In addition, infection rate of *Cryptosporidium* spp. by this technique in Iran was 12.3% (Tavalla *et al.*, 2017). However, the percentage of current results was compatible with the percentage of infection in Egypt. In addition, the current results were different with other studies in China (Jian *et al.*, 2014) they found infection rates of *Cryptosporidium* spp. in dogs by using PCR were 6.9% and in Poland (Piekara-Stepinska *et al.*, 2021), *Cryptosporidium* spp. was identified by nested PCR in canine fecal samples was 3.4%.

According to the phylogenetic tree, the *Cryptosporidium* spp. isolates in this study exhibited similarity to sequences deposited in GenBank, with the maximum homology of the gene sequences between the Iraqi *Cryptosporidium* spp. strains and strains worldwide being 99%–100% for dogs. The phylogenetic analysis results confirmed minimal differences between the Iraqi strains of *Cryptosporidium* spp. and those from other countries. This genetic variation may be due to differences in the area size of the reference sequence and in the geographical areas where the isolates were collected. Various methods were used for the genetic analysis, including nPCR-based gene sequencing of partial or complete genes. The results of this study align with studies that have reported the existence of genetic variation and phylogenetic relationships among *Cryptosporidium* spp. populations worldwide thought these isolates to be adaptable for both zoonotic and anthroponotic transmissions, by direct or indirect contact. The current findings were in an agreement with by previous studies (Gharieb *et al.*, 2018; Adam *et al.*, 2019; Al-Yasary and Faraj, 2021). Overall, the present study highlighted the presence and prevalence of this protozoan in dogs living in this city, as well as identifying two important species *C. canis* and *C. parvum*. One of them is *C. parvum* which is a zoonotic species that plays a vital role in zoonotic infections.

It can be concluded that the current study highlighted the presence and distribution of the infection by *Cryptosporidium* species in dogs in this part of Iraq, where the infected males recorded the highest infection rate compared with infected females. The results also revealed that the infection rate was high in dogs in the age less than one year old. In addition, the infected dogs in rural areas had the highest infection rate compared with those in urban areas. The findings of the PCR and DNA sequencing techniques added significant knowledge to the present study, recording two important species of genus *Cryptosporidium*, included; *C. canis* which is a specific species infecting dogs, and *C. parvum* which is a zoonotic species

between human and dogs. Importantly, the outcomes of the molecular techniques consider the first molecular investigation that has identified these two species in dogs living in Babylon province, Iraq. Thus, further research may be useful to understand the epidemiology of this infection and procedures for its control.

HIGHLIGHTS

- This is the first study that identified *Cryptosporidium Canis* in dogs in Babylon province.
- This study considers the first contribution to the knowledge of a zoonotic transmission for *Cryptosporidium parvum* infecting dogs in Babylon Province
- These findings have highlighted the role of dogs in transmitting zoonotic species of *Cryptosporidium* to humans, which could have epidemiological significance on public health.
- The findings have significantly given obvious indicators to health authorities to investigate the most important measures to limit the spread of dogs, especially stray dogs, in the urban and rural areas of this city.

ACKNOWLEDGMENTS

The authors did not receive any funds for this study. The authors are thankful to the Department of Parasitology, College of Veterinary Medicine, Al-Qasim Green University, Iraq for providing the necessary facilities of this study.

NOVELTY STATEMENT

The first genetic investigation and Microscopic diagnosis of *Cryptosporidium* species infecting dogs also, the zoonotic species of *C. parvum* in dogs in Babylon city, Iraq.

AUTHOR'S CONTRIBUTION

Both authors equally contributed to complete this research.

ETHICS APPROVAL

This study was approved by the ethics committee of the College of Veterinary Medicine/Al-Qasim Green University according to the letter (No.1152 in 18/7/2023) before beginning work on the present study; we contacted animal owners and obtained their permission to include their animals' data in the current study.

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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