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Molecular identification and phylogenetic study of *Toxocara canis* in domestic and stray dogs from Karbala, Iraq

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ABSTRACT. The ascarid nematode, *Toxocara canis*, which causes Toxocariasis in dogs, is a member of the Toxocaridae family and genus *Toxocara*, and that is accidentally infects humans. The study was conducted to investigate the presence of *T. canis* in dogs in Karbala, Iraq. The study extended from January to October, 2023. This project is the first of its kind in Iraq to molecularly detect the parasite in stray dogs in Karbala and to report its prevalence in domestic and stray dogs by utilizing conventional PCR and fecal flotation techniques while controlling for risk factors. One hundred (50 stray and 50 domestic) dog fecal samples were included. The findings revealed that 26 (26%) of the total dog fecal samples were tested positive using the PCR method. In details, the infection rate was 10% in domestic dogs and 42% in stray dogs. Moreover, the rate was 9.3 in adult dogs and 38.5% in puppies. The results showed that age, lifestyle, and infection rate all showed significantly ($p < 0.01$) correlated relationships in dogs. The correlation between *T. canis* infection rates and sex was not statistically significant ($p > 0.01$). The PCR-based sequencing of *T. canis* isolates indicated a high percentage of similarities with strains from different geographical regions. The study findings indicate higher rates of *T. canis* infection in adult and puppy stray dogs, explaining the extent of the infection among domestic dogs and stray dogs, using the latest laboratory methods for diagnosis PCR with the use of the phylogenetic tree to show the extent of similarity with species found in neighboring countries. The study findings are important by giving initial epidemiological data for future studies and control of the parasite.

Keywords: dogs; *Toxocara canis*; PCR.

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Introduction

Toxocariasis is a disease caused by the parasite Toxocara canis that infects domestic animals (Overgaauw, 1997). Some parasites may cause ocular and visceral larval migrations in animals and humans, especially roundworms (Hotez, Fenwick, Savioli, & Molyneux, 2009). The field of veterinary medicine dealing with dog breeding and related disorders is ever-evolving and requires a unique approach because vital information, including the incidence of various diseases and basic data, is lacking, particularly in Iraq (Saaed & Alsarhan, 2022). Canine-related diseases can spread orally and feces-wise through contact with canines, raw vegetables, contaminated hands, or soil that harbors infective eggs (Wolfe & Wright, 2003). Toxocariasis typically manifests as an acute clinical disease in young puppies. A loss of condition, poor growth, and occasionally delayed puberty are the typical clinical indications. The disease can be accompanied by the presence of worms, either in vomit or feces. Moreover, constipation, vomiting, diarrhea, and flatulence are some other clinical signs of the illness (Hosin, 2008). The eggs have pitted shells and are thick and dark brown in color, which leads to the ultimate diagnosis (Rashid, Aziz, Ali, Kakarash, & Marif, 2022). PCR could be considered a diagnostic tool for Toxocariasis (Smith & Noordin, 2006). A small number of studies conducted in Iraq's middle and southern cities have revealed the existence of Toxocara infections in dogs. *T. canis* infection was found to be highly prevalent in stray dogs (25.98%), according to a study conducted in the province of Kirkuk (Hassan & Barzinji, 2018). In the past few years, the number of stray dogs in Karbala Province has increased, particularly in parks and residential areas. This could have a serious negative effect on public health as zoonotic illnesses such as Toxocariasis, which can be spread by these dogs (Santarém, Magoti, & Sichieri, 2009). Due to the high incidence of infection and subsequent harm to human health; it appears that owners of domestic dogs are connected to the spread of *Toxocara spp*. worms (Hade, Saadedin, Al-Amery, & Ibrahim,

2018). This is likely because deworming programs are not being implemented. The research gaps There is difficulty in dealing with samples due to the difficulty of dealing with stray animals, in addition to the necessity of dealing with samples quickly and accurately during molecular examination. The tendency of most previous studies in Iraq was to adopt microscopic diagnosis due to the difficulty of providing samples and materials and their high prices, which resulted in less accurate results, in addition to many studies that did not show the extent of the importance of the quality of life of dogs and the comparison of the impact on the quality of life between stray dogs and domestic dogs and infection rates. The study was conducted to investigate the presence of *T. canis* in dogs in Karbala, Iraq. The study contributed in findings is important because they provide initial epidemiological data for future studies and control of the parasite.

Materials and methods

Samples collection

Fife grams of feces from each of 100 dogs (50 domestic and 50 stray cats) were collected in Karbala, Iraq. The samples of feces were put in a container labeled with date, age, date, and sex and transferred to the University of Al-Qadisiyah, College of Veterinary Medicine, Laboratory of Microbiology, for investigation. The study was carried out from January to October 2023.

The flotation method

Five grams of well-homogenized feces were thoroughly mixed with fifty milliliters of NaCl-saturated solution. Using a piece of gauze, the content was filtered. A coverslip-covered tube was used. Nematode eggs were then allowed to float and come into contact with the coverslip for duration of 25 min. After removing the coverslips horizontally and placing them on the glass slides, they were inspected at a power of 10x (Cringoli, Rinaldi, Maurelli, & Utzinger, 2010).

Molecular study

Molecular study was conducted for detection of *T.canis* in dogs and construction phylogenetic relationship. This study was including:

PCR and isolation of DNA

The whole sequence of the *Toxocara canis* isolate 5.8S rRNA and internal transcribed spacer 2 (ITS2) RNA gene from GenBank (KF577856.1) was utilized to design the primers in this work. The ITS-2 region is important and ideal for determining the history of the development of sequence maps (Al-Fatlawi & Al-Fatlawi, 2022). Bioneer Company, Korea, which is shown in Table 1, supplied these primers.

Following the guidelines provided by the company, the genomic DNA was extracted using the kit from Geneaid, located in Korea. The extracted DNA was examined using a device known as a nanodrop spectrophotometer (THERMO, USA-made). By measuring the absorbance at (260/280 nm), this instrument determines the concentration and purity of DNA. As stated in Table 2, this technique was implemented in accordance with guidelines. The results of the PCR were then examined using agarose gel electrophoresis.

Table 1. PCR primers of *Toxocara canis*.

Primer name	Sequence 5' 3'	Product size
Forward	CTCGAGTCGACGAAGTATGTAC	
Reverse	AATTGGGCCGCCCATCATAC	200 bp
Target	ITS2	

Table 2. The PCR thermocycler constructed reaction conditions for ITS2 of *T. canis*.

PCR step		Celsius (°C)	Seconds (s)
One-Repeat-Denaturation-1		95	180
	Denaturation-2	95	35
39 repeats	Annealing	52	35
	Extension-1	72	35
One-repeat-Extension-2		72	300

Amplicon sequencing and analysis

From the positive PCR results, ten samples were chosen for Macrogen DNA Sanger sequencing in South Korea. Once the generated sequences were obtained, they were trimmed from noisy signals and then deposited in the gene bank. After getting the relevant accession numbers, they were being analyzed for phylogeny and compared with other global strains as follows: The DNA sequencing study (phylogenetic tree analysis) was carried out using Molecular Evolutionary Genetics Study Version 10 (Mega X) and multiple sequence alignment analysis based on Clustal W alignment analysis. These were contrasted with global strains to find similarities and differences from NCBI-Blast. The software from the following reference (Stecher, Tamura, & Kumar, 2020) was used to conduct this analysis.

Statistical analysis

The Statistical Analysis System (SAS, 2018) program was used for Chi-square of (0.05 and 0.01 probability) (Al-Ukaelii & Al-Shaeb, 1998).

Results

Microscopic examination results

The *T. canis* egg was observed using light microscopy. As seen in Figure 1, eggs are spherical to ovoid in shape, dark brown in color, and have thick pitted shells.

Results of molecular study

Out of 100 fecal samples examined by PCR, the total infection rate was 26 (26%) in *T. canis* (Table 3).

In details, the infection rate was 10% in domestic dogs and 42% in stray dogs. Moreover, the rate was 9.3% in adult dogs and 38.5% in puppies. The results showed that lifestyle and infection rate showed significantly ($p < 0.01$) correlated relationships in dogs (Table 4).

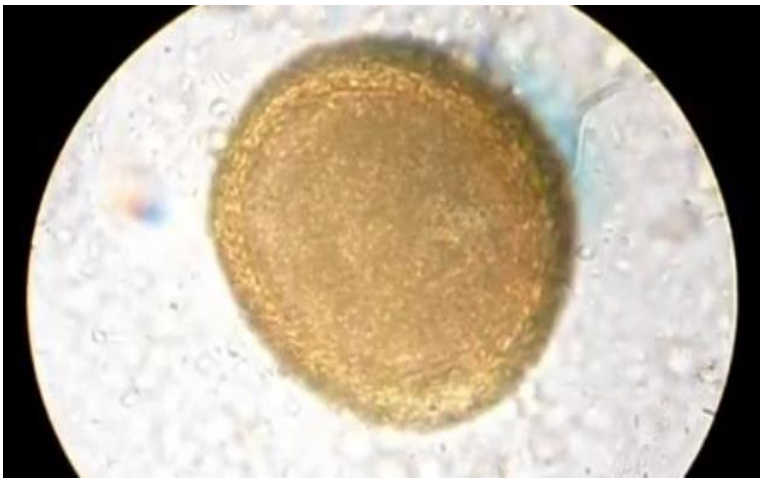

Figure 1. *Toxocara canis* eggs detected by light microscopy at (100X).

Table 3. Total rate of *Toxocara canis* infection in dogs by PCR. .

Host	No. samples	Positive	Percentage %
dog	100	26	26

Table 4. Percentage of *Toxocara canis* infection in dogs according to Lifestyle.

Lifestyle	No. samples	Positive	Percentage %	χ^2	p-value
Stray	50	21	42		
Domestic	50	5	10	17.602 **	0.0001
Total	100	26	26		

**($p \leq 0.01$).

**($p \leq 0.01$) = highly significant.

In Table 5, the age and infection rate showed significantly ($p < 0.05$) correlated relationships in dogs.

48 fecal samples collected from male dogs and 52 samples collected from female dogs were examined for *T. canis*; 12 male dogs (25%) and 14 female dogs (26.9%) were confirmed to be infected with *T. canis* (Table 6). There was no significant difference ($p > 0.05$) in the infection rate between the sexes.

Results obtained by PCR technique

Figure 2 shows positive amplicons (1-10) of *T. canis* targeting partial region of 5.8S rRNA and ITS2 (size= 200 bp). NC is H2O-negative control. M is molecular marker (100- 1500 bp) from Genedirx (South Korea).

Result of DNA sequencing and phylogenetic tree construction

Using data from the local *T. canis* nucleotide sets used in the current investigation, they were NCBI-evaluated and verified. Local isolates of *T. canis* were entered into the NCBI-Genbank database and assigned Genbank accession numbers. Sequences of local strains were aligned with reference strains of *T. canis* that had previously been documented in Genbank. The sequencing and phylogenetic results are shown in Table 7.

Table 5. Effect of age on the infection rate *Toxocara canis* in Dogs.

Age	No. samples	Positive	Percentage %	χ^2	P-value
Puppy	57	22	38.5		
Adult	43	4	9.3	11.469 **	0.0017
Total	100	26	26		

**($p \leq 0.01$).

**($p \leq 0.01$) = highly significant.

Table 6. Effect of sex on the infection rate *Toxocara canis* in Dogs.

Sex	No. of samples	Positive	Percentage %	χ^2	P-value
Male	48	12	25		
Female	52	14	26.9	0.519 NS	0.689
Total	100	26	26		

NS: Non-Significant.

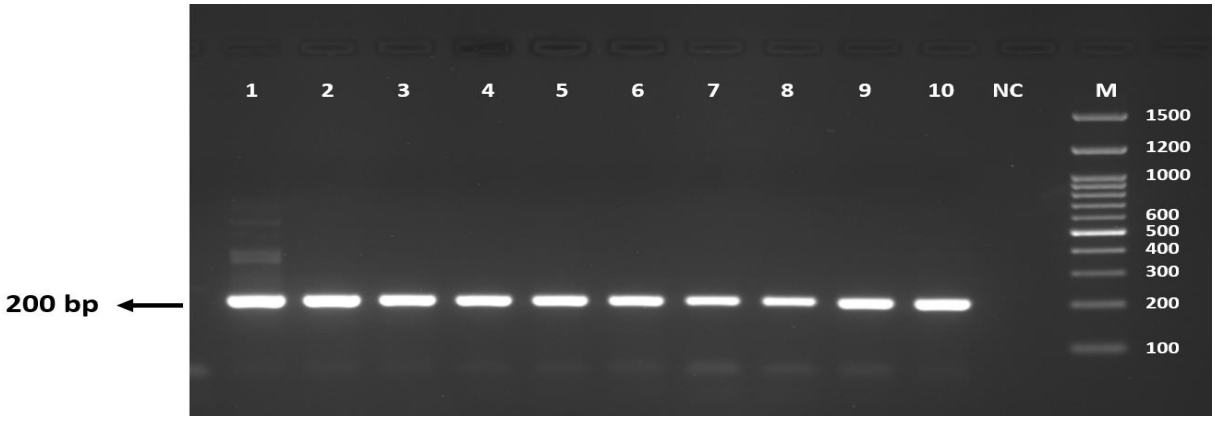

Figure 2. Agarose gel electrophoresis image (1.5% agarose) for 5.8S rRNA and ITS2 (size= 200 bp).

Table 7. The NCBI-BLAST homology Sequence identity (%) in local *Toxocara canis*.

Accession numbers (Current)	Identical to	Global Accession number	Country	Identity (%)
OR623059	*Toxocara canis*	MF592391	Iran	97.93
OR623060	*Toxocara canis*	LC762621	Germany	97.93
OR623061	*Toxocara canis*	OM876370	China	97.93
OR623062	*Toxocara canis*	KJ777156	India	97.05
OR623063	*Toxocara canis*	AB110034	Japan	100
OR623064	*Toxocara canis*	KJ777156	India	99.16
OR623065	*Toxocara canis*	MT939441	Iran	100
OR623066	*Toxocara canis*	OP185363	Iran	100
OR623067	*Toxocara canis*	KF577855	Iran	100
OR623068	*Toxocara canis*	KF577855	Iran	100

DNA sequencing and phylogenetic tree of *Toxocara canis*

The tree scale was according to the number of substitutions per site. Rooted tree was based on Ascaridoidea using MEGA11(Figure 3).

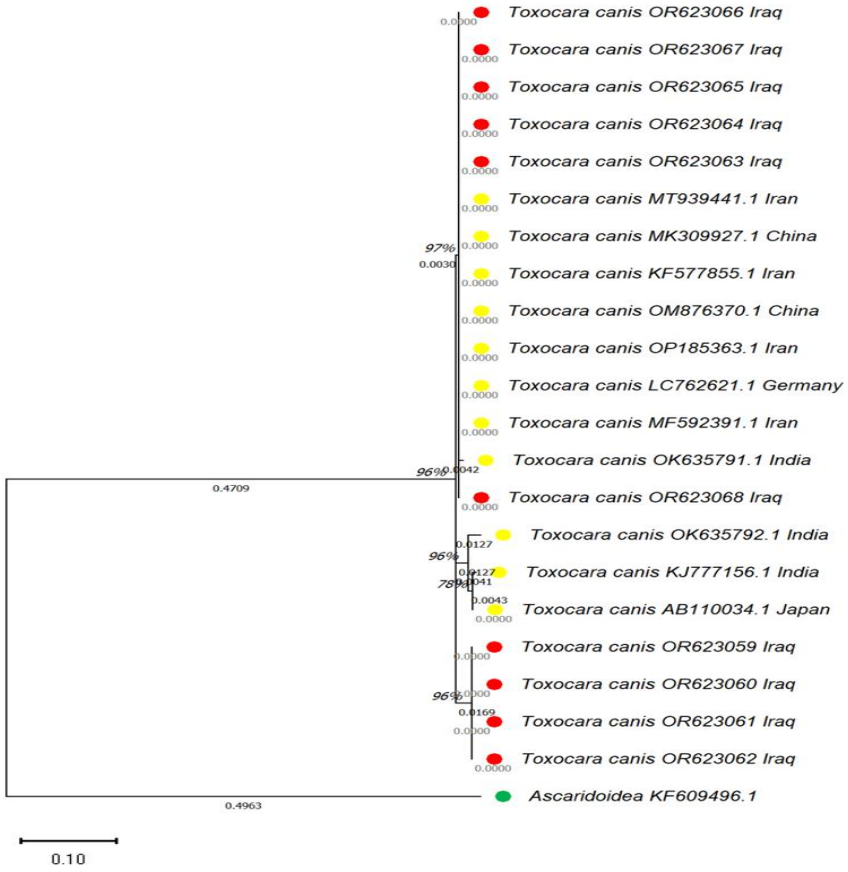

Figure 3. Evolutionary analysis of *T. canis* (red circles referred to the currently identified sequences while yellow circles referred to other global sequences).

Discussion

The identification of parasite eggs is a common diagnostic method for canine Toxocariasis (Mohamad, Azmi, & Noordin, 2009). This study used molecular detection to examine the *T. canis* parasite in dogs. For the first time, the molecular biology of *T. canis* in dogs from various regions of the Iraqi province of Al-Karbala was studied. The prevalence of Toxocara in dogs was 26%. The prevalence was higher than that of Rubel, Zunino, Santillán, and Wisnivesky (2003), who found 22% in the middle-class area. This is similar to studies conducted in Kirkuk City by Hassan (Hassan & Barzinji, 2018), who found 25.98%. According to Awadallah and Salem (2015), Malaysia is 11.9 and Egypt is 5.38%, respectively, which were higher than other studies. The frequency of *T. canis* was less than that recorded in Baghdad in 2021 by Khalil, Al-Taii, and Mahmood (2023), who discovered infection rates of 65.5 and 42.4% in 90 stray and 125 house dogs, respectively.IN Mosul, Iraq *T. canis*; 40.47% (Arsalan, Daham, Al-Obaidi, & Sulaiman, 2006).

No seroprevalence variations were found between male and female canines based on sex, which is similar to the findings published by Tinoco-Gracia, Barrenas-Serrano, López-Valencia, and Tamayo-Sosa (2007). On the other hand, other data indicated that seroprevalence was higher in male dogs (Regis et al., 2011). It is also crucial to keep in mind that pregnant female dogs retain larval stages in the tissues of their bodies, which subsequently infect their pups (Quiroz, 1999). Age differences were seen in the current study infection rate. According to studies conducted in Europe and India, the prevalence of *T. canis* was higher in younger dogs and cats in Europe (38.5%) and India (38%), than in older dogs and cats in Europe (14.7%) and India (Claerebout et al., 2009; Barutzki & Schaper, 2013).the result disagree with result (Jarad, Abbas, & Aȧiz, 2019) older dogs more in Al-Diwaniya province, Iraq. Dogs that are older than six months old and have stronger immune systems seem to have a lower risk of infection compared to younger age groups (Ilić et al., 2021).

One of the most important reasons for the existence of some differences in proportions from previous studies is the difference in sample numbers, in addition to a difference in the nature of the tests used, in addition to a difference in the environment and lifestyle of dogs in other countries and the methods used in controlling and preventing diseases that affect dogs. *T. canis* may occasionally go undetected; however, tissue larvae are possible, mostly in females. Nonetheless, the organism may reactivate during pregnancy and infect the fetus (Overgaauw & Knapen, 2013) either prior to or following delivery via the placenta or lactation (Telmo et al., 2015; Okada, Ooi, & Taira, 2021).

The Sequencing and phylogenetic results showed four *T. canis* isolates (OR623065, OR623066, OR623067, and OR623068) had a close relationship with NCBI-Blast *T. canis* of Iran (MT939441, OP185363, and KF577855) with 100% identity, whereas other isolates (OR623059) were related with 97.93% identity. Two *T. canis* isolates (OR623062 and OR623064) had a close relationship with NCBI-Blast *T. canis* of India (KJ777156) with 97.5 and 99.16% identity. The isolate (OR623060) had a close relation with NCBI-Blast *T. canis* of Germany (LC762621) with 97.93% identity; isolate (OR623061) had a close relation with NCBI-Blast *T. canis* of China (OM876370) with 97.93% identity; and isolate (OR623063) had a close relation with NCBI-Blast *T. canis* of Japan (AB110034) with 100% identity.

The highly identity can be due to it clustering in the same nodule, which explains a tight relationship between them, and this may be because these countries are adjacent to one another and situated along a single geographic line, allowing for migration of the paratenic hosts like rodents and birds between these countries. These results that the research reached by showing the extent of the infection, the nature of the infection, and methods of diagnosing it have an important role in controlling it and reducing its seriousness, which helps veterinary and health authorities in their work by providing a database that facilitates their work.

Conclusion

The current investigation indicates that dogs residing in the study location had a rather prevalent case of *T. canis*. The lifestyle of the animal and the incidence of infection in dogs were highly correlated. Age and the frequency of illnesses in dogs, however, did not correlate. The current study is of utmost importance because the results showed the high rates of infection, the factors associated with it, the strains, and their closeness to the strains in neighboring countries. This will be a basis for controlling the disease and a data set for researchers in the future.

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