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Molecular Detection and Prevalence of the *Toxocara Cati* Parasite in Household Cats Breeds in the Al-Anbar Province-Iraq

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Toxocara cati is a globally prevalent parasitic roundworm that affects cats. It is a member of the Ascarididae family, which includes one of the most common intestinal parasites. The aim of this study was to investigate the prevalence of *Toxocara cati* in a total of 100 individuals from two major breeds from Al-Anbar (50 Shirazi and 50 Himalayan breeds). Therefore, this is the first parasite investigation as well as molecular characteristic analysis to be conducted in Iraq on household cats. After conducting a comprehensive examination, the clinical indicators exhibited by these animals were reported. In order to examine parasite eggs under a microscope, we collected faeces from each animal. A small sample of faeces was also subjected to molecular analysis. Blood samples were also used to study the effects of this parasite on eosinophils. PCR-based approaches that employ genetic markers from the nuclear and mitochondrial genomes have emerged as viable substitutes due to their sensitivity, specificity, speed, and effectiveness. Our investigation found that infestation rates, according to the molecular method, were 31% (15 in Shirazi and 16 in Himalayan), which was similar to microscopy results. Subsequently, domestic cats residing in urban Al-Anbar exhibit an elevated prevalence of *T. cati*. Therefore, it is crucial to develop effective methods for identifying and eliminating *T. cati* parasites in domestic cats, while simultaneously prioritising public education on animal and human health.

Keywords: Shirazi, *Toxocara cati*, *ITS-2* gene, Himalayan, zoonotic parasite.

Introduction

Domestic and stray cats are the definitive hosts of *Toxocara* spp., which are considered zoonotic parasites of public health and veterinary concern [1, 2]. This parasitic nematode can infect feline breeds. Besides cats, it can infect other animals, including dogs, wild felids, and foxes [3, 4].

This pathogen has a life cycle that begins with the ingestion of infective eggs, which are present in contaminated soil or faeces of infected cats. These eggs can hatch and develop into adult worms and grow up to 10 cm in length, with females capable of producing hundreds of thousands of eggs daily. Moreover, human infections are more commonly reported in children, especially those of low age [5, 6]. Keeping cats as pets, touching and playing with these animals, geophagy by children, free entry of dogs and cats into farmland and public parks, and non-compliance with sanitation in eating non-washed vegetables are among the most important risk factors associated with toxocariasis [7].

Furthermore, their infection can be detected in cats using clinical elements, microscopy and molecular paths. Clinical signs of *Toxocara cati* infection may include vomiting, diarrhoea, abdominal pain, weight loss, and poor body condition [8]. A microscopic study could provide information about the morphological characteristics of *T. cati* in feline breeds. This

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can involve examining faecal samples from infected cats for the presence of *T. cati* eggs [9]. A molecular study could provide information about the genetic makeup of this parasite in household cats. This might involve amplifying specific genes from *Toxocara cati*, such as the second internal transcribed spacer (*ITS-2*) region of ribosomal DNA [10,11].

Overall, the prevalence of endoparasites in cats in Europe has been found to vary between 20% and 40% [12]. Also, several studies reported that stray or free-ranging outdoor cats have a higher frequency of parasites than indoor-keeping cats. For instance, in Greece, stray cats were 8.8 times more likely to be infected with Toxocariasis when compared to owned cats. Similarly, the infection rate of *T. cati* was 2.7 times higher for cats living outdoors in comparison to those staying indoors [13,14].

As previously reported, *T. cati* infection is common in some Iraqi cats. [15] found a 40% prevalence in Mosul. [16] reported a 12.9% incidence in Baghdad. However, there is no molecular data about this disease in Iraq, generally, as well as no epidemiological study of it among household cats in Al-Anbar province; therefore, this research was conducted based on clinical, microscopic and molecular characterizations.

Material and Methods

Ethics approval

The author confirms that the present work complied with all necessary protocols and has received approval from the College of Veterinary Medicine at the University of Baghdad. The researcher collected the blood samples from the animal in accordance with the earlier mentioned ethical standards.

Study area, sampling, and design

The study area was in Al-Anbar, the western province of Iraq. The samples were obtained during the period from October 2022 to January 2023. A total of 100 faecal and blood samples were taken from Shirazi and Himalayan cats (fifty samples from each breed). Veterinary clinics and cat owners provided these samples. Six to eight grams of cat faeces are deposited in a plastic container with the sample number, date, age, sex, and breed noted. For parasitological microscopic examination, three to five grams of each sample are collected, and the rest is frozen for molecular analysis.

Blood examination (eosinophil cell)

About 1 ml amount of blood is obtained from the cat's vein **(**cephalic vein**),** with a sterile needle and syringe for haematological testing (differential white blood cell count). Next, another glass slide is used to spread the drop of blood out into a thin, equal layer on the first slide. The smear is left to dry naturally. The blood smear is dried and then stained with Giemsa dye, so that the various types of white blood cells may be better distinguished under the microscope. Based on the overall quantity of white blood cells, the percentage of each kind can be determined [17].

Microscopic investigation

Microscopic examination of faeces is a widely used diagnostic tool to identify the presence of parasite eggs or larvae in the gastrointestinal tract of animals, such as cats infected with *Toxocara cati*. The flotation method is a common diagnostic method used to detect parasite eggs. In this method, mix a little excrement with a flotation solution (saturated NaCl) in a test tube. Thus, parasite eggs float to the surface of the flotation solution due to its higher specific gravity. Coverslips are placed over the tube and removed after a few minutes to be placed on microscope slides [18,19].

Molecular study

This method is employed for the extraction of genomic DNA from faeces samples, both fresh and frozen, was described. So, this study is used the PrestoTM Stool DNA Extraction Kit, which a commercially available product that has been created by *Geneaid* Biotech Ltd. The kit employs a patented DNA extraction buffer and spin column technique in order to obtain substantial quantities of DNA that exhibit superior quality, rendering it appropriate for subsequent applications including PCR, qPCR, sequencing, and genotyping.

The utilisation of genetic markers located in the *ITS-2* region of ribosomal DNA has demonstrated its efficacy in the identification of individual eggs at the species level through the process of PCR amplification. Consequently, this particular region has exhibited significant advantages in the field of parasitic worm research [20].

To determine the identification of *Toxocara cati*, a total of one hundred DNA samples were subjected to the usual polymerase chain reaction (PCR) protocol. In this study, a set of two distinct oligonucleotide primers were developed for each *ITS-2* region gene of *T. cati*. These primers were utilised in a typical polymerase chain reaction

(PCR) assay, which employing pure DNA extracted from *T. cati* eggs. Besides, we have designed these primer sequences, for the *ITS-2* region of the cati 5.8S ribosomal RNA gene, in depending NCBI rules, and the *ITS2*, namely the partial sequence in the 5' to 3' direction (Table 1). Amplification of the ribosomal *ITS-2* gene of *Toxocara cati* was under a specific condition of the PCR thermocycles, which accompanied by the following stages (Table 2). Additionally, the PCR data was collected following the amplification steps. The 10 µl of amplified sample was put on a 1.5% agarose gel with 3 µl /100 ml ethidium bromide and 80 V for 1 hour. A loading buffer and a DNA size marker were also added. Products were visualised using a UV transilluminator and photographed with a digital camera; positive results were determined when the sample's DNA band base pairs were the same as the desired product size.

Phylogenetic sequence analysis.

The sequencing data underwent processing utilising the GenBank database. The data were subjected to analysis using MEGA X software

TABLE 1. *Toxocara cati* 5.8S ribosomal RNA gene and internal transcribed spacer 2

Band size	Sequence (5->3)	Primer
232 bp	TGGTGCATTCTTTCGCAACG	Forward primer.
	GCCGATGACGTTACCTCCAA	Reverse primer

TABLE 2. Steps of the PCR condition and thermocycle

No.	Steps	Temperature	Time	Cycles
1	Initial denaturation	94 ^{0}C	10 minutes	
2	Denaturation	94 ^{0}C	1 minute	
3	Annealing	58 ^{0}C	20 Second	40
4	Extension	72 ^{0}C	24 Second	
5	Final extension	72 ^{0}C	10 Minute	

(Version: MEGA, 11.0.11) in order to construct a phylogenetic tree, with the aim of identifying genetic similarities between the investigation of T. cati and isolates from around the world.

Statistical Analysis

It involved the use of SPSS (version 25) to analyse the data. The Chi-Square Test (specifically the McNemar Test) was employed to assess the correlation between all parameters. The findings indicated significant results at a significance level of $p \leq 0.05$.

Results and Discussion

Clinical findings

Our study found the percentage of cats showed clinical symptoms of infection (15%). Our clinical findings revealed variability in the manifestation of several symptoms, with the predominant symptom being diarrhoea, which was the most frequently documented sign. The remaining indicators exhibited irregularity, including symptoms such as overall debilitation, emesis, conjunctivitis, and some neurological manifestations. These findings are aligning with the results of previous investigations wherein it was reported that of cats infected with *T. cati* had intestinal symptoms [21].

Microscopic Eggs detection in faeces.

The results of microscopic examination indicated that 31 samples out of a total of 100 samples of domesticated cats' faeces found to be positive for the presence of this parasite worm eggs, that is considered as moderate in their rate. More importantly, the prevalence of *Toxocara cati* eggs in Shirazi cats was 30% (15 out of 50), whereas in Himalayan cats was 32% (16 out of 50). Also, when viewed it under a microscope at a magnification of 40X, these eggs stand out for their round shape, jagged edges, and robust walls (Figure 1).

The present study's results agreed with a recent study investigated the coprological detection of *Toxocariasis* in the residential areas of the Kurdistan region of Iraq. The findings revealed that the prevalence of this parasite among stray cats was 47.62%, which is about four times higher than the infection rate observed in indoor cats, standing at 5.5% [22]. In addition, a recent comparative investigation was conducted to examine the prevalence of intestinal parasites in fecal samples obtained from both domestic and stray cats residing in Baghdad city. The study revealed a relatively low infection rate with 1.65% [23]. It likely that geographical variables and differences in detection methods may be account for the observed variations in *T. cati* prevalence among these studies.

Based on the findings of [24], it has been observed that the occurrence of *Toxocara* infection in feline is higher among stray cats that do not receive veterinary attention in comparison to cats that are owned by individuals. Furthermore, the previous data demonstrated a wide spectrum of prevalence rates, ranging from 5.45% to 67.5% in feral and free-ranging feline, as well as from 1.6% to 30.4% in domesticated cats [25].

Eosinophilia finding (differential WBC counts)

Blood smear findings in our study declared 9 (4 Shirazi and 5 Himalayan) of the infested cats had elevated eosinophil counts. So, eosinophilia were found to be significantly elevated based on the slide counted in this investigation.

T. cati egg-related to clinical symptoms and eosinophilia.

The analysis of faecal samples at a microscopic level aimed to identify parasite eggs, so the results indicated a strong link between prevalence rate and an increase of eosinophils in the blood for Shirazi and Himalayan cats, with P-values of 0.001 and 0.002, respectively. Furthermore,[15] [26] both reached of corresponding conclusions in their respective animal investigations, wherein they observed the presence of leucocytosis of their cases. Also, a comparison was made between the clinical symptoms that were seen in animals with *Toxocara cati* and the prevalence rate that was found by looking eggs at faeces from Shirazi and Himalayan cats under a microscope. Moreover, our study found that cats showing clinical symptoms of infection (15 out of 100) had a notably increased occurrence of parasite eggs in their faecal samples (P = 0.004 and 0.016) (Table 3).

Molecular detection of T. cati eggs by PCR

The polymerase chain reaction (PCR) method was used to look at 100 DNA samples, which taken from feline faeces. The results showed that a 232-base pair segment of the *ITS-2* gene of *T. cati* eggs was amplified in 31 of these samples. It showed that 16 samples (32%) from the Shirazi breed and 15 samples (30%) from the Himalayan breed were positive for *T. cati* (Figure 2).

When compared to more traditional assays, it appears that using PCR technology to detect *Toxocara* eggs in faecal samples is appropriate and accurate method. Moreover, all the data from microscopical process passed the PCR threshold of 232 base pairs from faecal samples of household cats (Shirazi and Himalayan breeds). Also, there was no statistically significant difference between the results obtained using the molecular method (infection rate, 31%) and those obtained using the microscopic technique (infection rate, 31%) (P = 1.000) (Table 4).

Our study's outcomes are consistent with those of a study [27]. In that study, ribosomal DNA from *T. cati* was found in the faeces of infected cats. So, the prevalence of infection among the feline population was determined to be 47%. In contrast, [28] reported opposite results in southwestern Iran when using microscopy and molecular techniques to detect and classify soil contamination, which caused by *Toxocara* eggs. In particular, their study showed that *Toxocara* eggs were in 30.4% and 33.8% of the soil samples, which they found by looking at the samples with a microscope and PCR technique. Furthermore, several studies confirmed that *Toxocara* spp. eggs can be distinguished from the eggs of other closely related and/or physically similar adult helminths by using PCR-based approaches that utilise the *ITS1* and *ITS-2* sections of rDNA [29,30].

More importantly, in the current study, all the data obtained from the microscopic procedure successfully met the PCR threshold of 232 base pairs. This is agreement with a study conducted by [31], when they have observed that *T. cati* was present in all 36 faecal samples from cats that tested positive under copro-microscopic examination. This study also agrees with a previous study that showed that the *ITS-2* region gene was confirmed as a useful genetic marker [32,33,34].

Molecular approaches utilizing ribosomal and mitochondrial markers have likely been developed to facilitate the accurate detection and diagnosis

of various species within the Ascarididae family, particularly during the larval and egg stages. This is particularly useful as these stages might provide difficulties in terms of morphological identification.

Furthermore, the results of our microscopic study were identical to those of our PCR experiment, but the process of identifying samples was quite complicated. This is may be due to morphological changes, as the identifying eggs extracted from frozen faeces presented considerable hurdles and required the knowledge of a highly skilled operator. Besides, PCR high sensitivity and specificity have a huge significance for the detection of infertile eggs. In spite of this, it may be used to spot diseased people or fertilised eggs. For instance, several studies not found *T. Cati* under the microscope method, but it detected by PCR technique [35,36]. It is also important to note that there are a number of factors which can prevent the eggs of this parasite from being expelled in their usual form. These factors include an extended period in which the eggs do not undergo embryonic development and intermittent excretion in the presence of obvious infections [37]. Given the above, we can conclude that routine microscopic testing of cat faeces is necessary to confirm the presence of an intestinal *Toxocara cati* infection [38]. Also, PCR may be suggested as an alternative in situations where a thorough analysis of parasites cannot be undertaken due to insufficient faecal samples.

Phylogenetic tree analysis

Moreover, we have conducted analysis of the phylogenetic tree in order to facilitate the comparison. The investigation analysed four sequence data sets of *T. cati* isolates from two Shirazi and two Himalayan breeds. All of the acquired sequences were subsequently submitted to the NCBI, and deposited it under accession number Sq3 (OR594359.1), Sq4 (OR594360.1), Sq5 (OR594361.1), Sq6 (OR594362.1). The results indicate that these isolates exhibit homology and similarity with isolates from various regions globally. Notably, the isolates showed a high similarity with Iran (LC700102.1), followed by India (KJ777157.1), China (Kyoto KY003083.1), Kazakhstan (OQ975261.1), and France (MT341306.1) (Figure 3).

The observed high degree of similarity between the two breeds can plausibly be attributed to the occurrence of random mating events among individuals belonging to both breeds. Furthermore, apart from the importation and commercial trading of these breeds across nations, the role of genetic diversity in the survival and adaptability of parasites becomes crucial when the parasite environment undergoes changes. Consequently, the precise analysis of this variation becomes relevant for conducting studies on various aspects such as the pathogenesis, epidemiology, population biology, taxonomy, and evolutionary biology of parasites [39].

Conclusions

This research is considered as first molecular study in Iraq and Al-Anbar province that used the *ITS-2* region gene as a useful molecular marker in different cat breeds, which can serve as a successful genetic marker with *T. cati* egg identification. The current study showed high percentage of infestation of this pathogen among all cat breeds in Al-Anbar area. PCR results of *T. cati* egg showed a high incidence in Shirazi (32%) to Himalayan (30%). Additionally, the molecular and microscopic data did not differ significantly among cat breeds. Also, our clinical and haematological data showed similar effects to other causes, especially in the gastrointestinal tract. The phylogenetic sequence results confirmed close relation among *ITS-2* gene of our breeds samples and samples from worldwide due to the open borders state, which means high level of transfer disease to our country from outside. For successful control strategies and to reduce the potential risks associated with zoonotic infections, educate pet owners whose animals may come into contact with stray animals.

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Authors contributions:

All Authors designed the entire work and worked simultaneously to collect data and statistically analyse it.

Conflict of interest:

All authors declare that they have no conflicts of interest.

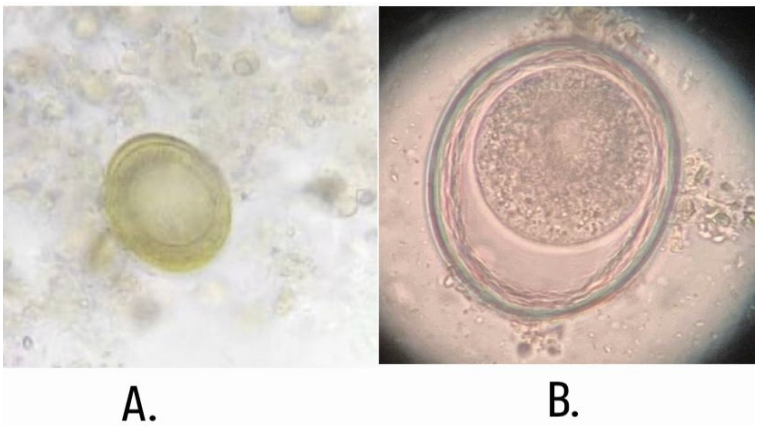

Fig. 1. *Toxocara cati* eggs detected under microscopic using two Flotation methods, A: examination with 40X magnifi*cati*on, B: examination with 100X magnification.

TABLE 3. Evaluation of the clinical signs and eosinophils findings in relation to microscopic eggs detection of *T. cati* egg presence in faecal cat breeds.

Test Statistics			
Cat breeds		**Microscopic eggs detection and Clinical signs**	**Microscopic eggs detection and Eosinophils results**
Shirazi	Number	50	50
	P-value	0.004 (Significance)	0.001 (Significance)
Himalayan	Number	50	50
	P-value	0.016 (Significance)	0.002 (Significance)

*For Chi square tests a significance level of p 0.05.

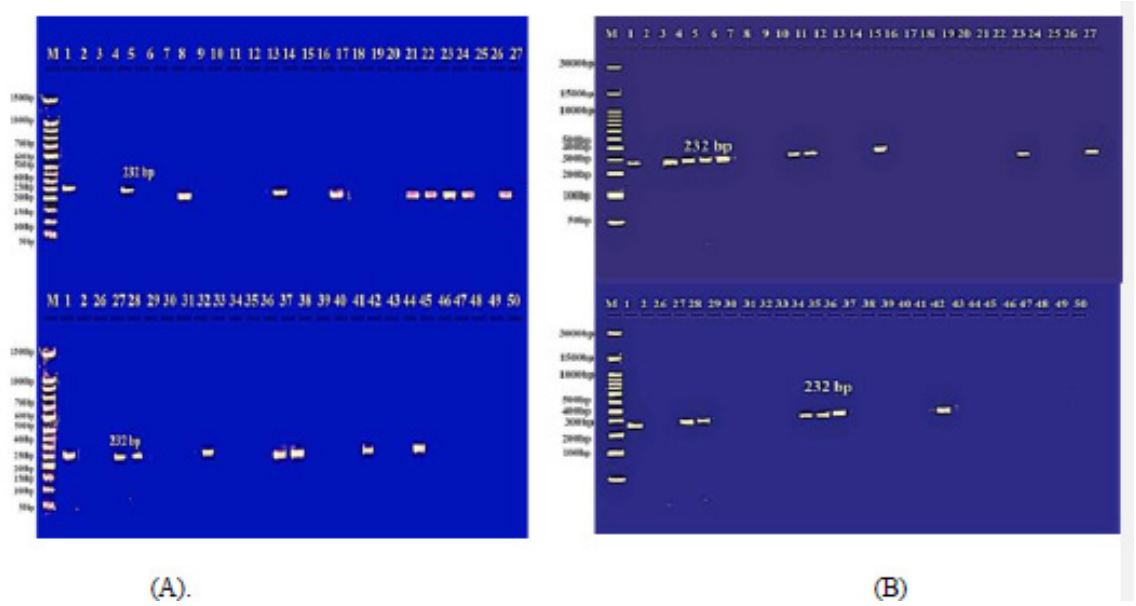

Fig. 2. Agarose gel picture appears the PCR product bands with molecular weight of 232bp. (M) refers to (3000 bp) DNA ladder, (1) positive control, (2) Negative control, and (3-50) PCR results of faeces samples.
A- PCR results of Shirazi breed.
B- PCR results of Himalayan breed.

TABLE 4. According to cat breed: The correlation between PCR and Microscopic eggs detection results.

Cat breeds	PCR	Microscopic eggs detection	
		Positive	Negative
Shirazi	Positive	16	0
	Negative	0	34
Himalayan	Positive	15	0
	Negative	0	35
Test Statistics[a]			
Cat breeds		PCR and Microscopic eggs detection	
Shirazi	Number	50	
	P-value	1.000 (No Significance)	
Himalayan	Number	50	
	P-value	1.000 (No Significance)	

a = The Chi-Square Test (specifically the McNemar Test) was employed to assess the correlation between all parameters. The findings indicated significant results at a significance level of $p \leq 0.05$.

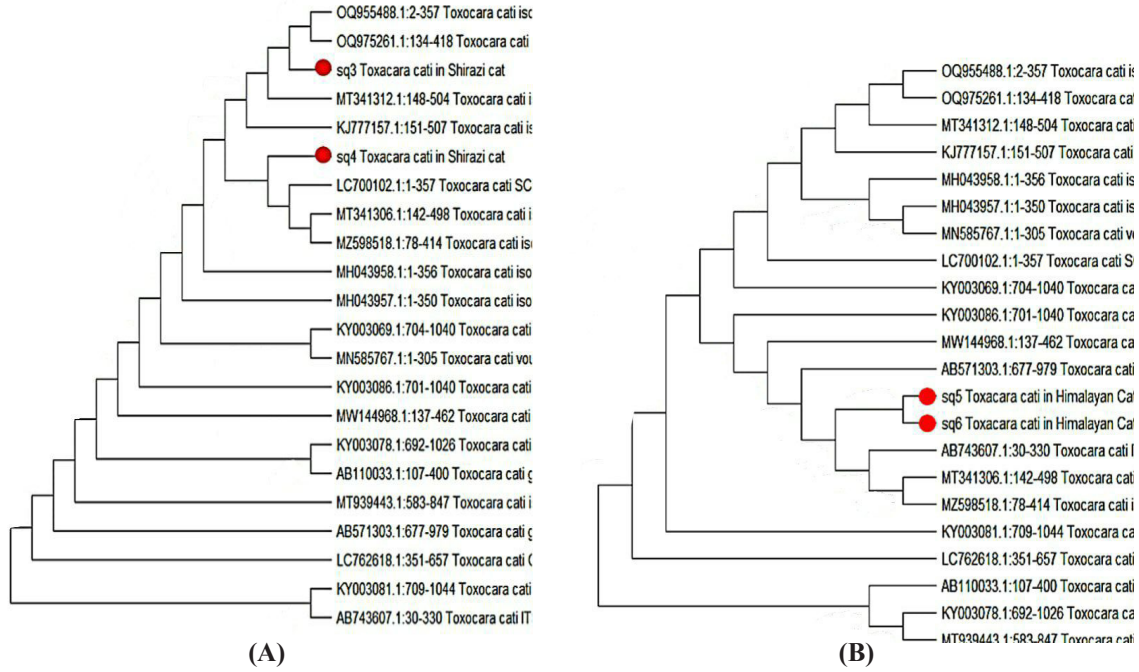

Fig. 3.The phylogenetic tree of *ITS-2* region of *T. cati*:
A- Shirazi breed;
B- Himalayan breed.

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الكشف الجزيئي وانتشار طفيلي توكسوكارا كاتي في سلالات القطط المنزلية في محافظة الأنبار-العراق

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التوكسوكارا كاتي هي دودة مستديرة طفيلية منتشرة عالميا تصيب القطط. وهو ينتسب الى عائلة الإسكارس، والتي تضم واحدة من أكثر الطفيليات المعوية شيوعا. كان الهدف من هذه الدراسة هو التحقيق في انتشار توكسوكارا كاتي فيما باجمالى ١٠٠ قطة منزلية من الأنبار (٥٠ سلالة شيرازي و٥٠ سلالة في جبال الهيمالايا). لذلك، يعتبر هذا هو أول تحقيق للطفيلي بالإضافة إلى تحليل الخصائص الجزيئية الذي يتم إجراؤه في العراق على القطط المنزلية. بعد إجراء فحص شامل، تم تسجيل المؤشرات السريرية التي ظهرت على هذه الحيوانات. من أجل فحص بيض الطفيليات تحت المجهر، قمنا بجمع البراز من كل. كما خضعت عينة صغيرة من البراز للتحليل الجزيئي. كما تم استخدام عينات الدم لدراسة آثار هذا الطفيل على الحمضات. ظهرت الأساليب القائمة على تفاعل البلمرة المتسلسل التي تستخدم الاشارات الجينية من الجينات النووية والميتوكوندريا كبدائل قابلة للتطبيق بسبب حساسيتها وخصوصيتها وسرعتها وفعاليتها. وجد تحقيقنا أن معدلات الإصابة، وفقا للطريقة الجزيئية، كانت ٣١٪ (١٥ في شيرازي و١٦ في الهيمالايا)، وهو ما كان مشابها لنتائج الفحص المجهري. بعد ذلك، تظهر القطط المنزلية المقيمة في مناطق الأنبار الحضرية انتشارا مرتفعا ل **التوكسوكارا كاتي**. لذلك، من الأهمية بمكان تطوير طرق فعالة لتحديد طفيليات **التوكسوكارا كاتي** والقضاء عليها في القطط المنزلية، مع إعطاء الأولوية في الوقت نفسه للتثقيف العام حول صحة والإنسان.

الكلمات المفتاحية: الشيرازي ، **توكسوكارا كاتي** ، جين *ITS-2* ، الهيمالايا ، طفيلي حيواني المنشأ.