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Original Article

Prevalence and Molecular Characterization of *Toxocara cati* Infection in Feral Cats in Alexandria City, Northern Egypt

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Abstract

Background: This study was performed to determine the prevalence and to identify precisely *Toxocara* spp., which infects feral cats in Alexandria, Egypt based on morphological and molecular approaches.

Methods: This cross-sectional study was carried out on 100 feral cats trapped from different areas of Alexandria during 2018. Adult male and female worms were recovered from small intestinal contents after euthanasia and dissection of cats. Distinct morphological features were initially determined using available keys, and then after amplification and sequencing of the mitochondrial NADH dehydrogenase subunit 1 (*nad1*) gene was carried out and phylogenetic trees were constructed.

Results: Forty out of 100 cats were infected with *Toxocara* spp. Intensity of infection ranged from 1 to 9 worms/cat, with a mean of 2.27±1.6. All isolates were confirmed as *T. cati* based on morphological features and the sequence of *nad1* gene. Results of the current study clearly show that Egyptian *T. cati* isolate examined herein is genetically similar to those recorded in other countries.

Conclusion: The current work revealed high prevalence of *T. cati* in feral cats in the study area. This is the first genetic study that confirms *T. cati* from feral cats in Egypt. In addition, it demonstrated the suitability and need of genetic markers such as *nad1* for identification of *Toxocara* spp. Furthermore highlights the public health importance of *T. cati* in Egypt.

Introduction

Toxocara are ascaridoid intestinal nematodes that their adults inhabits small intestine of various mammals. The most common species are *T. canis* which infects canines; *T. cati* which infects felines; and *T. vitulorum* which infects bovines. The first two species are known to be zoonotic parasites, capable of infecting humans (1, 2).

T. cati (Schrank, 1788) is one of the most common zoonotic gastrointestinal worms infecting cats "*Felius catus"* worldwide (3). Humans are usually infected through accidental ingestion of embryonated eggs from contaminated soil, ordinary people as well as some occupational groups are at high risk due to their direct contact with soil (3, 4).

Other members of the genus *Toxocara* i.e. *T. canis* and *T. malaysiensis* are also contributed in illness produced by zoonotic *Toxocara* in humans "i.e. larval migrans" (2). The clinical manifestations associated with toxocariasis are classified as visceral larva migrans, ocular larva migrans, covert toxocariasis, and may results in neurological toxocariasis (1, 5, 6). Moreover, inhalation of *Toxocara* spp. eggs that are suspended in air can produce allergic and respiratory manifestations (7). Recently, the epidemiological studies demonstrated that widespread prevalence of human toxocariasis in the world vary from 0.8 to 59.3% in different parts of the world (8, 9).

Feral and stray cats are often play a critical role in transmission of parasites especially those with zoonotic nature, recently this point becomes interesting and some studies were done to reveal such infections in Egyptian cats (10, 11).

Identification of parasites is an important point for understanding epidemiology and control of such infections of both medical and veterinary values (12), which is based solely on the morphological characters, identification and discrimination in-between *Toxocara* spp. might be confusing and mistaken (1, 13, 14). Felines are infected by *T. cati, T. malyesensis, Toxoascaris leonine,* and occasionally *T. canis* (13, 15), so the identification of Ascaridid nematodes infecting cats become more complicated and requires more precise tools. Molecular based methods in identification, discrimination of *Toxocara* spp. have been used successfully (16-19), suggesting that the mitochondrial genes might be good genetic markers for molecular identification and discrimination within *Toxocara* spp.

Thus, the current study designed to investigate the prevalence, and also to identify *Toxocara* sp. infecting feral cats in Alexandria city, Northern Egypt using morphological and molecular-based methods.

Material and Methods

Ethical approval

An ethical approval was obtained from Agricultural Research Center, Animal Health Research Institute (AHRI), Alexandria branch, with ensuring compliance of regulations of animal use in a human manner as recommended.

Study area

Alexandria city (31°12'N29°55'E) is the capital of Alexandria Province; extends along the coast of the Mediterranean Sea in north-central Egypt, about 114 miles (183km) northwest of Cairo (11, 20).

Samples collection

All over the year of 2018 (Jan-Dec); 100 feral cats were captured by trapping from different areas of the city. Captured cats were transferred to the laboratory of Department of Parasitology, Animal Health Research Institute, Alexandria branch. In the lab, general examination was done and each factor were recorded along with the date of capture and examination (11).

Parasitic materials

Individually, trapped cats were anaesthetized by an anesthetic drug "Xyla-ject®" (Xylazine Hydrochloride 23.3 mg, Adwia Company) by intramuscular injection at a dose 0.5 ml/10 kg body weight. After anesthesia, cats were humanely euthanized by chloroform. Their digestive systems were removed and the intestinal contents were examined for the presence of *Toxocara* nematodes. Recovered worms were washed extensively in 0.85% physiological saline and some were fixed in 70% ethanol alcohol and then identified through microscopical morphological examination. The adult *Toxocara* sp. worms were stored at -20 ˚C for molecular examination (17, 21). Mature male and female worms were identified according to the morphological features using available keys and descriptions (22, 23).

PCR study

Total genomic DNA was extracted using Qiamp DNA Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. PCR amplification was done to amplify a fragment (370 bp) of the NADH dehydrogenase subunit 1 gene (*nad1*) of mitochondrial (mt) DNA. The primer pair forward: 5´ TTCTTATGAGATTGCTTTT-3´ and reverse: 5´-TATCATAACGAAAACGAGG-3´ was used (24). The PCR mixture contained Emerald Amp GT PCR master mix (2X premix) 12.5 µl, PCR grade water 4.5 µl, forward primer (20 pmol) 1 µl, reverse primer (20 pmol) 1 µl and 6 µl template DNA; all in a 25 µl reaction volume. Each of the 35 PCR cycles consisted of 94 ˚C for 5 min. (primary denaturation), 49 ˚C for 30 sec. (secondary denaturation), 50 ˚C for 40 sec. (annealing), 72 ˚C for 45 sec. (extension); and 72 ˚C for 10 min. (final extension). PCR products were analyzed on a 1% agarose gel stained with ethidium bromide following electrophoresis and the DNA bands were visualized using a UV transilluminator and photographed. The PCR electrophoresis products were purified using a QIAquick PCR Product extraction kit (Qiagen Inc. Valencia CA) according to manufacturer's recommendations.

A purified PCR product was sequenced by using the same set of primer in the forward and/ or reverse directions on an Applied Biosystems 3130 automated DNA Sequencer (ABI, 3130, USA). Using a ready reaction Bigdye Terminator V3.1 cycle sequencing kit. (Perkin-Elmer/Applied Biosystems, Foster City, CA), with Catalog number 4336817.

Phylogenetic analysis

For the newly obtained sequence, a BLAST® analysis (Basic Local Alignment Search Tool) (25) was initially performed to establish sequence identity to GenBank accessions. Nucleotide sequences were compared with GenBank sequences using a BLAST search. Nucleotide sequence were assembled manually with the aid of CLUSTALW multiple alignment program (17, 26). Furthermore pairwise alignment was performed by Lasergene molecular biology software. Bootstrapped trees were constructed by MEGA6 (27). To construct a phylogenetic tree based on *nad1* mtDNA, homologues and selected related sequences that are deposited in the DDB/EBML/Genbank were obtained and used. These used parasites were *T. cati* (JF833958.1), *T. canis* (JF833955.1), *Parascaris equorum* (MF678786.1), *Ascaris suum* (HQ704901.1), *T. malaysiensis* (AJ937264.1) and *Ascaris lumbricoides* (KY045802.1). Alignments of the gaps were not counted, and 500 bootstrap replications were performed Amino acids sequences of the mitochondrial *nad1* gene were inferred using the universal codons table.

Statistical analysis

Statistical analysis was performed using SPSS (Chicago, IL, USA) 20.0 (Statistical Package for Social Science, IBM Corporation).

Results

In autopsied feral cats, 40% were infected with *Toxocara* sp. (Table 1). The intensity of infection ranged between 1 and 9 worms per cat, with a mean of 2.27±1.6. The collected *Toxocara* worms were initially identified as *T. cati* based on morphological features. Briefly, all examined specimens had broad cephalic alae giving a copra-like appearance. Female worms were distinguished by the presence of intrauterine eggs that are brown and pitted. Male nematodes had a curved posterior end with paired spicules and caudal papillae, showing a prominent point at the tail-end finger like process which was distinguishable from the straight-tailed females.

Table 1: Prevalence of *Toxocara cati* detected in feral cats in Alexandria city, Northern Egypt (No=100)

Factor	*No. of cats examined*	*Cats infected (No.)*	*Prevalence (%)*
Age			
Young ≤ 1 yr	70	37	37
Adult > 1 yr	30	3	3
Gender			
Female	60	30	30
Male	40	10	10
Total	100	40	40

A band of 370 bp was successfully produced for *nad1* gene (Fig. 1). Sequencing of the obtained product done and the sequence obtained from the Egyptian isolates in the current study was deposited in GenBank under the accession number MK692514.

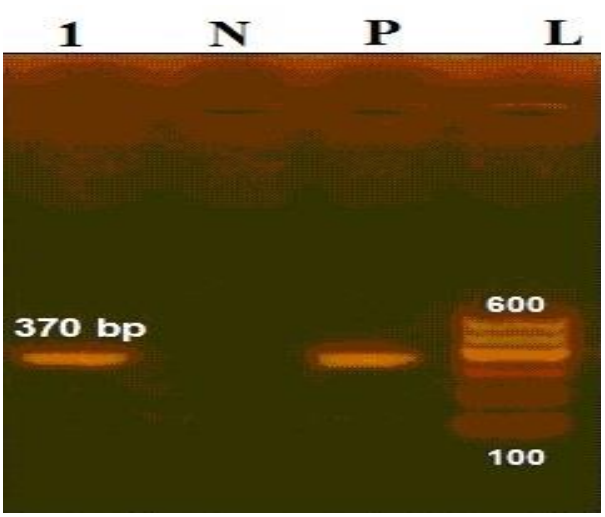

Fig. 1: Analysis of PCR product by electrophoresis on 1% agarose gel stained with ethidium bromid showed that amplification of *nad1* gene mtDNA. Lane 1: *Toxocara cati* Egyptian isolate, a positive band of 370 bp; lane N: negative control (no DNA); lane P: positive control (with DNA); and lane L: 100-600 bp molecular weight marker

Constructed phylogenetic tree (Fig. 2) showed that *T. cati* from stray cats of Egypt clustered with their respective reference strains of *T. cati*. Moreover, all *T. cati* aligned in one clad. *T. canis* (JF833966, AJ 920382, AJ920383) aligned in another group. *T. malysesnsis* (AJ937264) was distinct from both. The resulted sequence of the current work when compared using BLAST with those of *T. cati* deposited previously in GenBank showed 98.36-99.45% identity with *T. cati* from Iran (e.g. KC200232, KC200216, KC200214), 98.90 with *T. cati* from China (AM411622), and *T. cati* from Poland (KX963445) (Fig. 3). Variation between our newly obtained sequence and *T. cati* sequence from Iran (KC200232) was only in two nucleotide sites. This result confirmed the Egyptian isolate as *T. cati*.

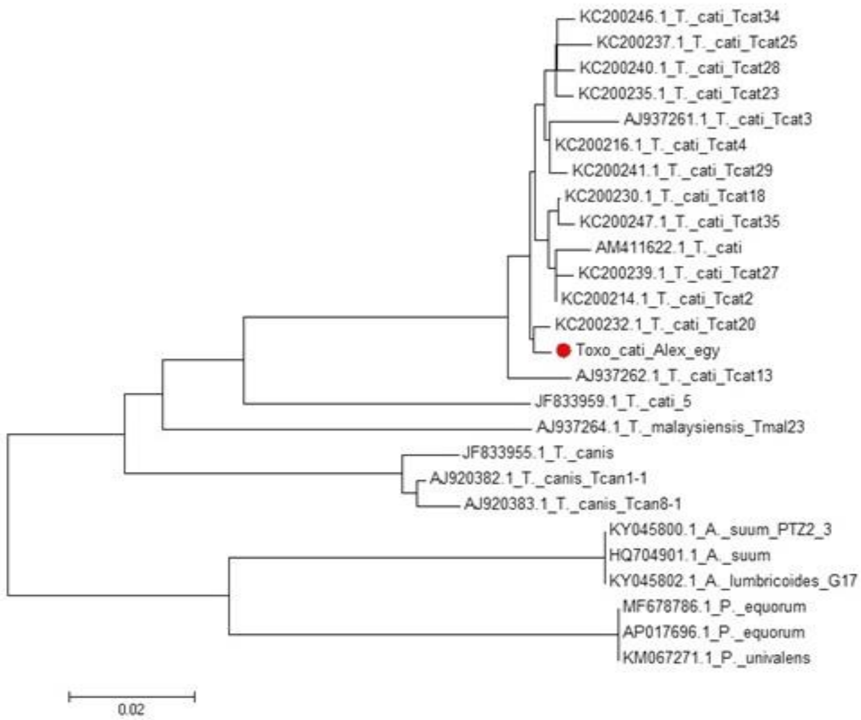

Fig. 2: Phylogenetic relatedness of the *nad1* gene. Maximum like hood uprooted tree generated after 500 bootstraps indicating clustering of the tested strain with *Toxocara cati* strains apart from other nematodes. Tree was constructed by using MEGA6

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26		
1		98.9	98.9	98.4	98.6	98.4	99.2	99.5	99.2	98.9	98.4	98.1	97.8	97.8	91.0	98.9	89.1	88.5	88.5	88.8	84.2	84.2	84.2	83.9	83.9	83.9	1	AM411622.1 T. cati
2	1.1		99.5	98.9	99.2	98.9	99.2	99.5	99.2	98.9	98.9	98.6	98.4	98.4	91.0	99.5	89.6	89.1	89.1	89.3	85.2	85.2	85.2	85.0	85.0	85.0	2	KC200232.1 T. cati Tcat20
3	1.1	0.5		99.5	99.7	99.5	99.2	99.5	99.2	98.9	99.5	99.2	98.4	98.9	91.0	99.5	89.6	89.1	89.1	88.8	84.7	84.7	84.7	85.0	85.0	85.0	3	KC200216.1 T. cati Tcat4
4	1.7	1.1	0.5		99.2	99.5	98.6	98.9	99.2	98.9	99.5	99.2	97.8	98.4	91.0	98.9	89.1	88.5	88.5	88.8	84.7	84.7	84.7	85.0	85.0	85.0	4	KC200246.1 T. cati Tcat34
5	1.4	0.8	0.3	0.8		99.2	98.9	99.2	98.9	98.6	99.2	98.9	98.1	98.6	90.7	99.2	89.3	88.8	88.8	88.5	84.4	84.4	84.4	84.7	84.7	84.7	5	KC200241.1 T. cati Tcat29
6	1.7	1.1	0.5	0.5	0.8		98.6	98.9	99.2	98.9	99.5	99.2	97.8	98.4	91.0	98.9	89.6	89.1	89.1	88.8	84.4	84.4	84.4	84.7	84.7	84.7	6	KC200240.1 T. cati Tcat28
7	0.8	0.8	0.8	1.4	1.1	1.4		99.7	99.5	99.2	98.6	98.4	98.1	98.1	90.7	99.2	89.6	89.1	89.1	88.5	85.0	85.0	85.0	84.7	84.7	84.7	7	KC200239.1 T. cati Tcat27
8	0.5	0.5	0.5	1.1	0.8	1.1	0.3		99.7	99.5	98.9	98.6	98.4	98.4	91.0	99.5	89.6	89.1	89.1	88.8	84.7	84.7	84.7	84.4	84.4	84.4	8	KC200214.1 T. cati Tcat2
9	0.8	0.8	0.8	0.8	1.1	0.8	0.5	0.3		99.7	99.2	98.9	98.1	98.1	90.7	99.2	89.3	88.8	88.8	88.5	84.4	84.4	84.4	84.2	84.2	84.2	9	KC200230.1 T. cati Tcat18
10	1.1	1.1	1.1	1.1	1.4	1.1	0.8	0.5	0.3		98.9	99.2	97.8	97.8	90.4	98.9	89.3	89.1	89.1	88.3	84.2	84.2	84.2	83.9	83.9	83.9	10	KC200247.1 T. cati Tcat35
11	1.7	1.1	0.5	0.5	0.8	0.5	1.4	1.1	0.8	1.1		99.2	98.4	98.4	90.4	98.9	89.6	89.1	89.1	88.8	84.7	84.7	84.7	85.0	85.0	85.0	11	KC200235.1 T. cati Tcat23
12	1.9	1.4	0.8	0.8	1.1	0.8	1.7	1.4	1.1	0.8	0.8		97.5	98.1	90.2	98.6	89.3	89.1	89.1	88.0	83.9	83.9	83.9	84.2	84.2	84.2	12	KC200237.1 T. cati Tcat25
13	2.2	1.7	1.7	2.2	2.0	2.2	1.9	1.7	1.9	2.2	1.7	2.5		97.3	91.0	98.4	88.5	88.0	88.0	88.8	84.7	84.7	84.7	84.4	84.4	84.4	13	AJ937262.1 T. cati Tcat13
14	2.2	1.7	1.1	1.7	1.4	1.7	1.9	1.7	1.9	2.2	1.7	1.9	2.8		90.4	98.4	89.9	89.3	89.3	88.3	84.4	84.4	84.4	85.0	85.0	85.0	14	AJ937261.1 T. cati Tcat3
15	9.8	9.9	9.9	9.8	10.2	9.8	10.2	9.9	10.2	10.5	10.5	10.8	9.9	10.5		91.0	87.2	86.6	86.6	90.4	86.6	86.6	86.6	83.6	83.6	83.6	15	JF833959.1 T. cati 5
16	1.1	0.5	0.5	1.1	0.8	1.1	0.8	0.5	0.8	1.1	1.1	1.4	1.7	1.7	9.9		89.6	89.1	89.1	88.8	84.7	84.7	84.7	84.4	84.4	84.4	16	Toxo_cati_Alex_egy
17	12.0	11.3	11.3	12.0	11.7	11.3	11.3	11.3	11.7	11.7	11.3	11.7	12.7	11.0	14.5	11.3		98.4	99.2	89.9	85.5	85.5	85.5	86.1	86.1	86.1	17	AJ920382.1 T. canis Tcan1-1
18	12.7	12.0	12.0	12.7	12.4	12.0	12.0	12.0	12.4	12.0	12.0	12.0	13.4	11.7	15.2	12.0	1.7		98.6	89.3	85.5	85.5	85.5	86.6	86.6	86.6	18	JF833955.1 T. canis
19	12.7	12.0	12.0	12.7	12.4	12.0	12.0	12.0	12.4	12.0	12.0	12.0	13.4	11.7	15.2	12.0	0.8	1.4		89.3	85.2	85.2	85.2	85.8	85.8	85.8	19	AJ920383.1 T. canis Tcan8-1
20	12.3	11.7	12.4	12.3	12.7	12.3	12.7	12.4	12.7	13.0	12.3	13.4	12.4	13.0	10.4	12.4	11.1	11.8	11.8		85.5	85.5	85.5	83.9	83.9	83.9	20	AJ937264.1 T. malaysiensis Tmal23
21	17.9	16.5	17.2	17.2	17.5	17.5	16.9	17.2	17.5	17.9	17.2	18.2	17.2	17.5	14.8	17.2	16.2	16.2	16.5	16.2		100.0	100.0	89.1	89.1	89.1	21	KY045802.1 A. lumbricoides G17
22	17.9	16.5	17.2	17.2	17.5	17.5	16.9	17.2	17.5	17.9	17.2	18.2	17.2	17.5	14.8	17.2	16.2	16.2	16.5	16.2	0.0		100.0	89.1	89.1	89.1	22	KY045800.1 A. suum PTZ2_3
23	17.9	16.5	17.2	17.2	17.5	17.5	16.9	17.2	17.5	17.9	17.2	18.2	17.2	17.5	14.8	17.2	16.2	16.2	16.5	16.2	0.0	0.0		89.1	89.1	89.1	23	HQ704901.1 A. suum
24	18.2	16.8	16.8	16.8	17.2	17.2	17.2	17.5	17.9	18.2	16.8	17.9	17.5	16.8	18.6	17.5	15.5	14.8	15.8	18.3	11.9	11.9	11.9		100.0	100.0	24	MF678786.1 P. equorum
25	18.2	16.8	16.8	16.8	17.2	17.2	17.2	17.5	17.9	18.2	16.8	17.9	17.5	16.8	18.6	17.5	15.5	14.8	15.8	18.3	11.9	11.9	11.9	0.0		100.0	25	AP017696.1 P. equorum
26	18.2	16.8	16.8	16.8	17.2	17.2	17.2	17.5	17.9	18.2	16.8	17.9	17.5	16.8	18.6	17.5	15.5	14.8	15.8	18.3	11.9	11.9	11.9	0.0	0.0		26	KM067271.1 P. univalens
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26		

Fig. 3: Sequence distance of the *nad1* gene of the tested nematode strain as generated by Lasergene software, showing identity range of *Toxocara cati* strains including the Egyptian one

Discussion

T. cati is an ascardid nematode of veterinary and public health importance (3), in order to reveal the prevalence, identify the species genetic structure and exact phylogenetic position of *T. cati* obtained from feral cats in Alexandria, Egypt. A survey was conducted, specimens collected, genomic DNA was extracted and *nad1* mtDNA gene was amplified and sequenced.

In the current study, almost 40% of cats were infected with *T. cati* . Such infection have been reported in other countries and Egypt (10, 11, 14, 16, 28) with different prevalence rates. For example, *T. cati* was reported from Iran with a prevalence (26.7-78%) (16, 29-31). In Hungary, Capári et al (32) detected *T. cati* with a prevalence rate (17.4%), from Greece Lefkaditis et al (33) reported it as (18.14%), and from Turkey (34) as (27.8%). In Egypt, *T. cati* was detected by much lower rate than that obtained current study (8-9%) (10, 11). The variation in *T. cati* prevalence among these studies might be contributed to geographic variation and the method of detection used. An important point should be highlighted when discussing the term "prevalence"; the size of sample, sampling procedures, nature of sample (coprological or necropsy), and other epidemiological and statistical factors may lead to variations among different studies results. So that, care should be paid in when discussing or comparing results of various studies. Herein, we mentioned the different prevalence rate of *T. cati* in different countries and regions to show the global spread of such infection.

In the current study, young cats were more infected than old one, this may disagree with some previous works (27, 35) who reported that age have no significance on the prevalence of *T. cati* infection. But this result was more logic due to the nature of complicated life cycle of *T. cati,* which results in infection of kittens via several ways (i.e. ingestion of embryonated eggs and/or getting infective larva from infected mother milk). Moreover, *T. cati* intensity ranged from 1 to 9 worms/cat, with a mean of 2.27, this in much agreement with the report of Changizi et al (36) (an average 3 worms/cat), but lower than results obtained by Sadjjadi et al (29) (an average 6.52 worm/cat). The intensity of infection is an important factor as egg production and subsequently to epidemiology of the disease is linked to number of worms/cat.

Morphological examination of specimens in the present work was in agreement with description of *T. cati* (15, 22, 23). In the past, for identification of a *Toxocara* species, the ordinary method of is determination of morphological characters. But this approach can have limitations in the differentiation of closely related species (2). Nowadays, molecular based techniques are used widely and are essential for precise identification of a parasite species (37). Recent researches on *Toxocara* spp. (2, 15, 17, 38, 39) have aimed to identify the species, determine the genetic make-up, the phylogenic relationship within closely-related species, and moreover to assist ordinary diagnosis. For example, although *T. canis*, *T. cati*, and the new *T. malaysiensis* can be confused with each other on the morphological level, they are genetically distinct (2, 17).

Mitochondrial genes for its conservation proved to be more suitable genetic markers for identification and discrimination of *Toxocara* spp. (19, 39, 40). Comparison of the newly obtained *nad1* sequence with other sequences of *Toxocara* on Genbank through BLAST confirms the isolate *as T. cati*, which clusters on the phylogenetic tree in one group with isolates from other areas of the world, proving that *nad1* gene mtDNA can be used as a genetic marker to identify and discriminate among *Toxocara* spp. Our successful amplification and sequencing of *nad1* gene *T. cati* supported previous work (14, 18, 19) suggesting *nad1* can be used as a gene marker for *Toxocara*. Molecular based methods are of great value to identify parasite species with certainty. For precise identification of a parasite, especially when morphological characters might be overlapping like in *Toxocara*, the ordinary microscopically methods should be accompanied by molecular ones.

Toxocara spp. are thought to be a host specific, this means cats are infected with *T. cati* and *T. malaysiensis*. While, dogs are infected with *T. canis*. Cross-infection or hybridization in-between these species (particularly *T. canis* and *T. cati*) was hypothesized by ordinary morphological examination. But absence of any molecular evidence of cross-infection support host specificity of *Toxocara*. Until a prove based on examination of mitochondrial cytochrome c oxidase subunit I (*cox1*) gene of *T. canis*, *T. cati*, *T. malaysiensis* and *Toxoascaris leonine* showed cross-infection of cats with *T. canis* and dogs with *T. cati* was shown for the first time (40). This confirm the need of molecular tools for parasites identification and superiority of mitochondrial genes like *cox1*, *nad1* for studying variation (inter-species, intra-species).

This is a preliminary molecular study on *T. cati* from Egypt in the future may be other molecular studies will goes on. Particularly that, human toxocariasis is a neglected parasitic zoonosis, with increased attention day after day due to its global expansion (28). Few reports on human toxocariasis were done in Egypt. Nevertheless, these reports showed significant and underestimated zoonosis, among 445 persons, 7.7% were seropositive for toxocariasis (41). In another report (42), out of 150 human sera samples examined for anti-*Toxocara* antibodies, 24% were positive. Stray animals is a serious problem, in maintain and distribution of zoonotic diseases like visceral larval migrans, particularly in developing countries like Egypt (43, 44). The exact number of feral and stray cats in Egypt is not known -to our knowledge- but it is assumed to be several hundred thousand, roaming streets even in villages. No strategic/national plan has been developed nor applied to control stray animals, including cats (43-45). Feral/stray cats population and overpopulation is a global problem, different strategies might be applied to overcome this problem (46). Nevertheless, recently about 118-150 million cats worldwide can serve as final hosts for *Toxocara* (47). Moreover, stray cats are more likely to be infected by *T. cati* than pet cats.

Conclusion

T. cati was detected in feral cats in Alexandria, Egypt with a high prevalence. The species identity was confirmed by molecular and phylogenetic methods. Further and in-depth studies on the prevalence, molecular, and genetic composition of *T. cati* and other *Toxocara* spp. in Egypt should be carried out, alongside with control programs for feral cats and stray animals.

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Conflict of interest

The authors declare that they have no conflict of interest. This study is a part of NMM requirements for Ph.D. in "Parasitology'.

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