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Original article

SEQUENCING AND PHYLOGENETIC ANALYSIS OF MITOCHONDRIAL *COX1* AND *NAD1* GENES IN *TOXOCARA CANIS* AND *TOXASCARIS LEONINA* ISOLATES FROM IRAN

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Summary

Valizadeh, M., F. Tahvildar Biderouni, S. R. Shahrokhi, M. Ghanimatdan & A. R. Nagahi, 2021. Sequencing and phylogenetic analysis of mitochondrial *cox1* and *nad1* genes in *Toxocara canis* and *Toxascaris leonina* isolates from Iran. *Bulg. J. Vet. Med.,* **24**, No 2, 251–260.

Toxocara canis and *Toxascaris leonina* are the most important ascaridoid nematodes of the family *Toxocaridae*. The present study was aimed to characterisation and analysis of genetic variation within and among *T. canis* and *T. leonina* isolates obtained from Iran by sequencing partial mitochondrial cytochrome c oxidase subunit 1 (*pcox1*) and partial NADH dehydrogenase subunit 1 (*pnad1*) genes. A total number of 134 adult nematodes belonging to *Toxocaridae* family were collected from stray dogs in Alborz province, Iran during 2015 and 2016. Polymerase chain reaction (PCR) was performed and products were sequenced. Sequences of two mitochondrial *cox1* and *nad1* genes were compared with other sequences in the GenBank, while multiple sequences alignment analysis was performed using the Bioedit and MEGA6 software and phylogenetic tree was plotted. For all isolates, amplicons of about 450 and 350 base pairs (bp) were successfully produced by PCR for *cox1* and *nad1*, respectively. All sequences of *T. canis* isolates from present study were 100% homologous across the *nad1* gene but not in the *cox1* gene. The results indicate that the PCR method based on sequence of *cox1* and *nad1* genes is a suitable technique for the differentiation of *T. canis* and *T. leonina* species and that mtDNA regions could be used as genetic markers for the identification and differentiation of *Toxocara* species.

Key words: *cox1*, Iran, mtDNA, *nad1*, *Toxocara canis, Toxascaris leonina*

INTRODUCTION

Toxocara canis, *T. cati* and *Toxascaris leonina* are the most important ascaridoid nematodes of the family *Toxocaridae*, causing accidental infection in humans as

their opportunistic host. However, *T. leonina* has a low zoonotic potential and low pathogenicity in humans (Overgaauw & van Knapen, 2013). *Toxocaridae* worms are among the most prevalent endoparasites in dogs and cats (definitive hosts), having a worldwide distribution which led to the high contamination of playgrounds, parks and households with *Toxocara* eggs (Robertson & Thompson, 2002).

Toxocariasis considered as a typical neglected disease, is caused by L2 larvae of *Toxocara spp.* with a number of clinical manifestations such as visceral larva migrans (VLMs), ocular larva migrans (OLMs), eosinophilic meningoencephalitis (EME), covert toxocariasis (CT) and neurotoxocariasis (NT) (Despommier, 2003; Macpherson, 2013).

Most metazoan mitochondrial (mt) genomes are circular and small (14–20 kb). The complete mt genomes of *T. canis* (14,332 bp), *T. cati* (14,029 bp) and *T. leonina* (14,310 bp) have been recently determined to provide novel mitochondrial DNA (mtDNA) markers for taxonomic and phylogenetic relationship analyses of *Toxocara* species (Li *et al.*, 2008a,b; Liu *et al.*, 2014). The mt genomes of the *Toxocara* species contain 12 proteins encoding genes (*nad1*, *atp6*, *nad2*, *cytb*, *cox3*, *nad4*, *cox1*, *cox2*, *nad3*, *nad5*, *nad6*, and *nad4L*), two ribosomal RNA genes (*rrnL* and *rrnS*), 22 transfer RNA (*trn*) genes, an AT-rich region and the non-coding regions (Wickramasinghe *et al*., 2009).

More efforts are spent on development of reliable diagnostic methods. Polymerase chain reaction (PCR) and sequencing of fragments of mitochondrial cytochrome C oxidase subunit 1 (*cox1*) and NADH dehydrogenase subunit 1 (*nad1*) genes, have been used for genetic analyses and identification of nematodes in dogs and cats (Oguz *et al.*, 2018; Jin *et al.*, 2019).

The present study was aimed to characterisation and analysis of genetic variation within and among *T. canis* and *T. leonina* isolates obtained from Iran by sequencing partial mitochondrial cytochrome C oxidase subunit 1 (*pcox1*) and partial NADH dehydrogenase subunit 1 (*pnad1*) genes. Using these sequences, the phylogenetic relationships of these parasites compared with isolates from different areas of the world based on the combined sequences of two mt gene fragments.

MATERIALS AND METHODS

Collecting of nematodes

A total number of 134 adult nematodes belonging to *Toxocaridae* family were collected from stray dogs in Alborz province, Iran during 2015 and 2016. Adult worms were taken from apparently healthy dogs 48 hours after taking levamisole, washed twice in phosphate-buffered saline (PBS), stained, and identified by using appropriate systematic keys.

DNA extraction, PCR enzymatic amplification and sequencing

DNA was extracted using phenol:chloroform:iso-amyl alcohol (25:24:1) followed by washing using chloroform: iso-amyl alcohol (24:1) and then stored at -20 ° C. Two mitochondrial genes (*cox1* and *nad1*) were amplified by polymerase chain reaction (PCR). The forward JB3 (5‘TTTTTTGGGCATCCTGAGGTTTAT 3‘) and reverse JB4.5 (5‘TAAA GAAAGAACATAATGAAAATG‘) primers were used to amplify a 450 bp portion of the mitochondrial *cox1* gene and the forward ND1F (5‘TTCTTATGAG

ATTGCTTTT 3‘) and reverse ND1R (5‘TATCATAACGAAAACGAGG‘) primers – to amplify a 350 bp fragment of the mitochondrial *nad1* gene. PCR reaction was performed with a final volume of 20 µL, containing 2 µL of template genomic DNA, 25 pmol of each primer and 10 µL of PCR premix (AmpliTaq Gold 360 Master Mix, cat. No. 4398876), which included 1.25 U Taq DNA polymerase, 200 µM of each dNTPs and 1.5 mM $MgCl_2$ in a thermocycler under following conditions: one cycle of 95 °C for 6 min (initial denaturation), followed by 35 cycles of 94 °C for 45 s (denaturation), 60 °C for 1 min (annealing), and 72 °C for 1 min (extension), and a final extension of 72 °C for 6 min. Double-distilled water (DDW) instead of template DNA was included in each set of PCR reaction as negative control. PCR amplicons were detected by 1.5% agarose gel electrophoresis and ethidium bromide gel staining. PCR products were sequenced by Bioneer company, South Korea. Five µL of PCR products were digested directly with 2 µL restriction endonuclease Rsa-1 (New England Biolabs (NEB), R0167S) for 6 hours at 37 °C. Restriction fragments of amplicons were detected using a 1.5% (w/v) agarose gel electrophoresis at 70 V for 45 min, ethidium bromide staining and visualized on a UV trans illuminator.

Sequences variability and phylogenetic analysis

Three amplicons of *T. canis* and two amplicons of *T. leonina cox1* and *nad1* genes were sequenced in Bioneer company, South Korea (https://www.bioneer.com). The sequences were compared with other sequences found in GenBank (https://www.ncbi.nlm.nih.gov/genbank/) using the BLAST (https://blast.ncbi.nlm.nih.gov/) and edited with Chromas® software. To illustrate the evolutionary relationship between the samples obtained in this study, multiple sequences alignment (MSA) was performed using BioEdit® software and phylogenetic tree was plotted using MEGA6® software with maximum likelihood method based on Kimura2-parameter model using bootstrap value with 1000 replication and a scale of 0.001.

RESULTS

For all isolates, amplicons of about 450 and 350 base pairs (bp) were successfully produced by PCR for *cox1* and *nad1*, respectively (Fig. 1). Sequences of the Iranian *T. canis* and *T. leonina* isolates *cox1* and *nad1* genes obtained in this study were submitted to GenBank and the accession numbers of these sequences are given in Table 1.

Cox1 gene

According to the multiple sequences alignment analysis of *cox1* gene, two samples of *T. canis* isolates were 100% homologous, while in the 369th position of third isolate sequence was adenine instead of Guanine (Fig. 2). The results indicate that the sequences of *cox1* gene of *T. canis1*, *T. canis2* and *T. canis3* isolates from this study were completely similar to Iran isolate (Accession no. KC293914) but different from another isolates from Iran (Accession no. KC293906 and KC293901) as well as isolates from China (Accession no. AJ920054 and AJ920056) in several nucleotides. *Cox1* gene phylogenetic tree results indicate that the sequences of the *T. leonina1* and *T. leonina2* isolates from present study were similar to isolates from Iran (Accession no. KC293927, KC2 93930, KC293934 and KC293935) and China (Accession no.

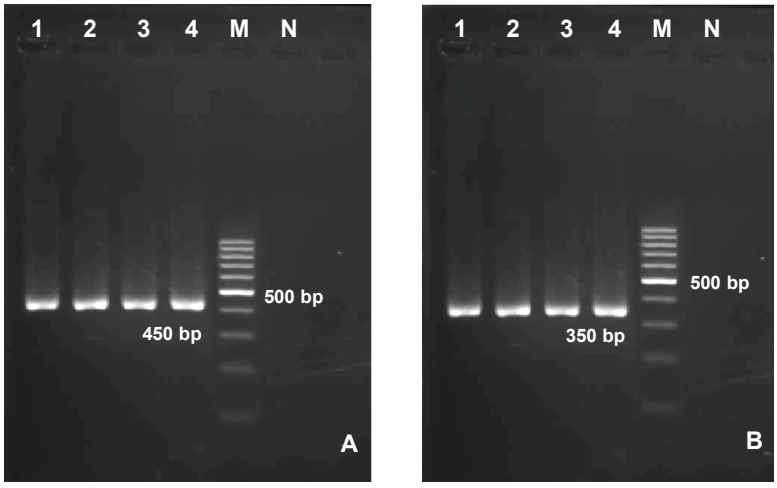

Fig. 1. PCR bands of *cox1* (~450 bp) (A) and *nad1* (~350 bp) gene (B) of *Toxocara* on 1.5% agarose gel. M: 100 bp DNA marker, N: negative control.

Table 1. *T. canis* and *T. leonina* isolates and their accession numbers of *cox1* and *nad1* genes of the present study

	Isolates	Accession No. *cox1*	Accession no. *nad1*
1	*T. canis1*	MK913431	MK913428
2	*T. canis2*	MK913432	MK913429
3	*T. canis3*	MK913433	MK913430
4	*T. leonina1*	MK591004	MK616562
5	*T. leonina2*	MK591005	MK616563

AJ920063 and JF780946) but different from isolates from Poland (Accession no. KX963448) and another isolate from China (Accession no. JF7800950) with 96% homology (Fig. 3).

Nad1 gene

According to the multiple sequences alignment analysis of *nad1* gene all sequences of *T. canis* isolates from present study were 100% homologous (Fig. 4). *Nad1* gene phylogenetic tree results show that the *T. canis* isolates of the present study were similar to isolate from Iran (Accession no. KC293915) with a homology of 100% but there were differences with two other isolates from Iran (Accession no. KC293920 and KC293922), as well as isolates from China (Accession no. AJ920385, AJ920386 and AJ920387). Also, *nad1* gene phylogenetic tree indicate that the *T. leonina* isolates from present study were similar to isolate from Iran (Accession no. KC293956) and different with isolates from China (Accession no. AJ893360) and Iran (Accession no. KC293955) (Fig. 5).

DISCUSSION

Ascaridoid nematodes have a significant adverse impact on human and animal health worldwide (Bethony *et al.*, 2006).

	1	2	3	4	5	6	7	8
1.MK913428.1 T.canis1* NAD1								
2. MK913429.1 T.canis2* NAD1	0.00							
3. MK913430.1 T.canis3* NAD1	0.00	0.00						
4. KC293915.1 T.canis IRAN	0.00	0.00	0.00					
5. KC293922.1 T.canis IRAN	0.00	0.00	0.01	0.00				
6. KC293920.1 T.canis IRAN	0.01	0.01	0.01	0.01	0.00			
7. AJ920385.1 T.canis CHINA	0.01	0.01	0.02	0.01	0.01	0.01		
8. AJ920386.1 T.canis CHINA	0.02	0.02	0.02	0.02	0.01	0.02	0.02	

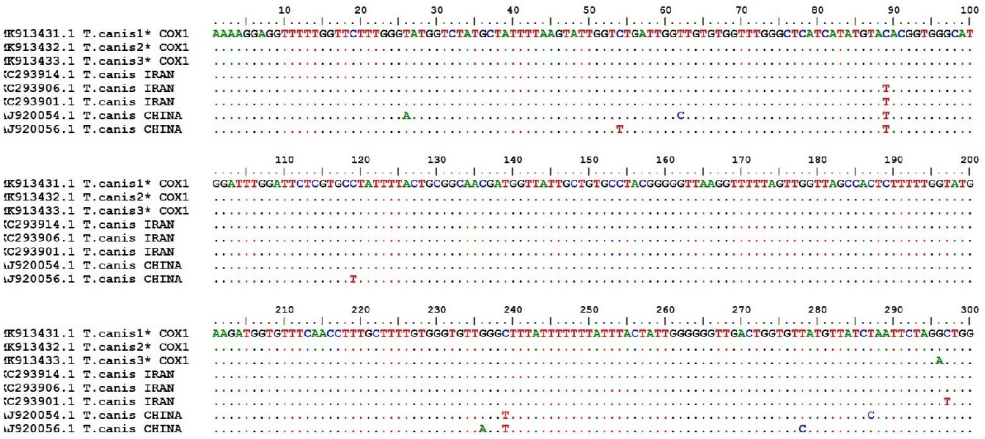

Fig. 2. Similarity degree and multiple sequencing alignment between *T. canis cox1* gene isolated from this study (marked with a star) and other isolates.

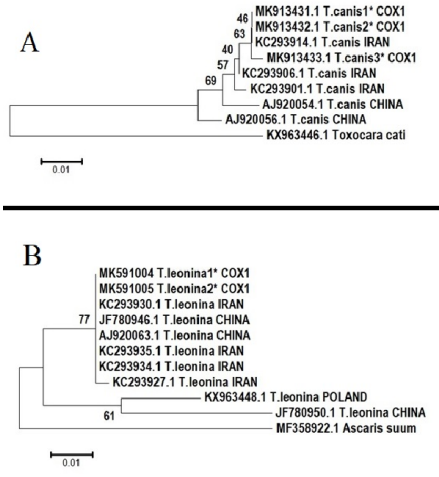

Fig. 3. Phylogenetic tree of *T. canis* (A) and *T. leonina* (B) *cox1* gene isolated from this study (marked with a star) with maximum likelihood method using the Kimura-2 parameter model. *Ascaris suum* was considered as the outside group.

	1	2	3	4	5	6	7	8	9
1.MK913428.1 T.canis1* NAD1									
2. MK913429.1 T.canis2* NAD1	0.00								
3. MK913430.1 T.canis3* NAD1	0.00	0.00							
4. KC293915.1 T.canis IRAN	0.00	0.00	0.00						
5. KC293922.1 T.canis IRAN	0.01	0.01	0.01	0.01					
6. KC293920.1 T.canis IRAN	0.01	0.01	0.01	0.01	0.00				
7. AJ920385.1 T.canis CHINA	0.01	0.01	0.01	0.01	0.00	0.01			
8. AJ920386.1 T.canis CHINA	0.01	0.01	0.01	0.01	0.00	0.01	0.01		
9. AJ920387.1 T.canis CHINA	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	

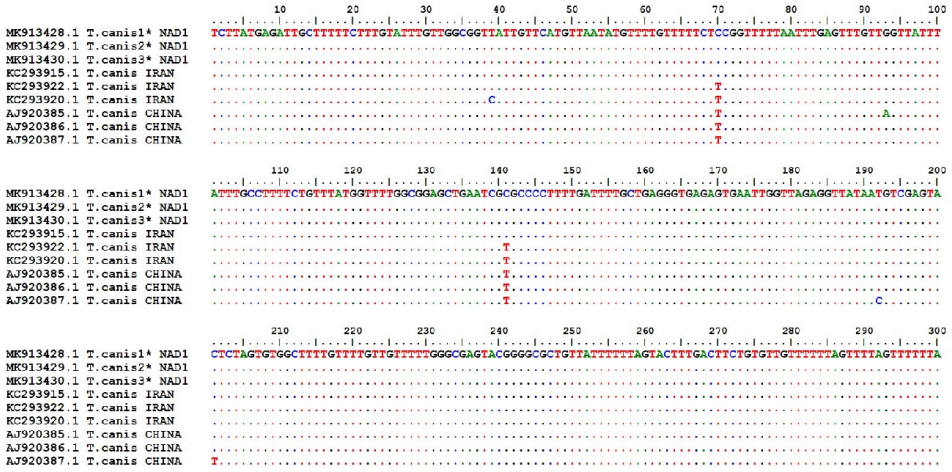

Fig. 4. Similarity degree and multiple sequencing alignment between *T. canis nad1* gene isolated from this study (marked with a star) and other isolates.

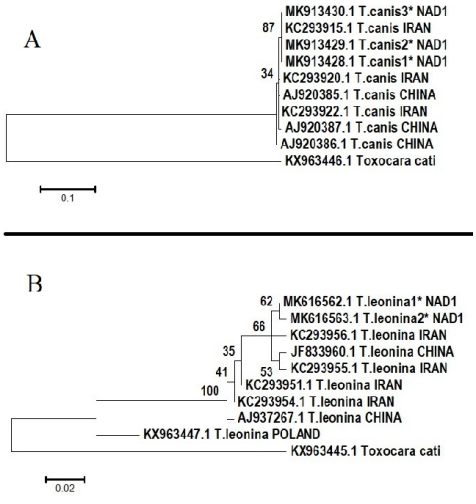

Fig. 5. Phylogenetic tree of *T. canis* (A) and *T. leonina* (B) *nad1* gene isolated from this study (marked with a star) with maximum likelihood method using the Kimura-2 parameter model. *Toxocara cati* was considered as the outside group.

T. canis (dog roundworm) is an important worldwide-distributed helminth parasite of dogs and *T. leonina* is a common ascaridoid nematode of dogs and cats (Becker *et al.*, 2012). The prevalence of *T. canis* in dogs ranged from 5.5% to 64.7% (Chen *et al.*, 2012) while *T. leonina* is more prevalent in wild and domestic carnivores (Okulewicz *et al*., 2012).

Zoonotic infections of nematodes are common in many developing countries. Fecal infection with *Toxocara* egg in carnivores has been reported in various regions of Iran. In many areas however, accurate information about the prevalence of *Toxocara* spp. does not exist. Most studies in Iran have been carried out on seroepidemiology of toxocariasis in apparently healthy people with no clinical signs, especially in <12-year-old children (Tahvildar Biderouni *et al.,* 2019). The high prevalence of human seropositive rate was reported in Fars and Tehran Provinces (Sadjjadi *et al*., 2001; Agin, 2012; Zibaei & Sadjjadi, 2017) moreover the high percentage of carnivore's infestation has been reported from East-Azerbaijan, Razavi Khorasan and Mazandaran Provinces in Iran (Daryani *et al*., 2009; Shemshadi *et al*., 2014; Hajipour *et al*., 2016).

Analysis of genetic sequence variability in parasites is widespread and has important outcomes for the molecular diagnosis, genetic structure, epidemiology, taxonomy, population biology as well as for the effective control of parasites (Stensvold *et al*., 2011). Mitochondrial DNA (mtDNA) has been used as a genetic marker to identify nematodes and cestodes and study genetic structures and phylogenetics. mtDNA is approved as a genetic marker for helminthic parasites epidemiological and genetics investigations (Wang *et al.*, 2011; Lin *et al.*, 2012; Wang *et al.*, 2013; Chang *et al.*, 2015; Poon *et al.*, 2017). *Toxocara* species could be differentiated from each other and from other ascaridoids by direct PCR based on *cox1* and *nad1* sequences. There are some reports on molecular differentiation of *T. canis* from other species based on PCR-linked restriction fragment length polymorphism (PCR-RFLP) assays analyses of rDNA, while no data are available from PCR-RFLP analyses of mitochondrial *cox1* and *nad1* genes. The data obtained by *in silico* analyses revealed that only one enzyme (MboII) is appropriate for PCR-RFLP on the *cox1* gene and there is not any suitable enzyme for PCR-RFLP on the *nad1* gene (Mikaeili *et al.*, 2017).

A few studies on the *cox1* and *nad1* sequence analysis of *T. canis*, *T. cati* and *T. leonina* has been done before (Li et *al.*, 2008a; Mikaeili *et al.*, 2015; He *et al.*, 2018). He *et al.* (2018) reported that *cox1* gene sequencing is enough to accurately distinguish and identify *T. cati*. The intra-specific sequence variations within *T. cati* were 0–3.6% for *cox1* (He *et al.*, 2018) Similarly Li *et al.* (2008b) described sequences variation in three mitochondrial DNA regions among and within *T. canis*, *T. cati*, *T. malaysiensis*, *T. vitulorum* and *T. leonina* in order to define genetic markers for their specific identification and differentiation. the intra-specific sequence variations were 0.2–3.7% for *cox1* and 0–2.8% for *nad1*, the inter-specific sequence differences were 7.9–12.9% for *cox1* and 10.7–21.1% for *nad1*. Phylogenetic analyses revealed that *T. malaysiensis* was more closely related to *T. cati* than to *T. canis* (Li et *al.*, 2008a).

This study was designed for analysis of genetic variation among and within

isolates obtained from stray dogs of Karaj, Iran and comparison with data available from other isolates found in GenBank. Based on multiple sequences alignment, there was sequence variation within and among the *cox1* gene of *T. canis1*, *T. canis2* and *T. canis3* isolates from this study and *T. canis* isolates from Iran (Accession no. KC293906 and KC293901) and China (Accession no. AJ920054 and AJ920056). These three isolates were identical and exhibiting 100% homology with an isolate from Iran (Accession no. KC293914). Sequence variations within *T. canis* isolate from this study were 0–0.3% for *cox1* and 0% for nad1. However, the sequence variations among *T. canis* isolate from this study and other isolates found in GenBank were 0–2.5% for *cox1* and 0.6–1.1% for *nad1*.

T. leonina isolates from present study showed 100% homology with isolates from Iran (Accession no. KC293927, KC293930, KC293934 and KC293935) and China (Accession no. AJ920063 and JF780946) across the *cox1* gene and 100% homology with isolate from Iran (Accession no. KC293956) across the nad1 gene. sequence variations within *T. leonina* isolate from this study were 0% for both *cox1* and *nad1* genes. However, the sequence variations among *T. leonina* isolate from present study and other isolates found in GenBank were 0–6% for *cox1* and 1–10% for *nad1*.

In a study performed in Iran, Mikaeili *et al.* (2015) noted that mitochondrial DNA regions could be used confidently for the identification of the nematode helminths. They reported that seven isolates of *T. leonina* collected from dogs in Iran did not differ in *cox1* gene from an isolate of *T. leonina* collected from a grey wolf (*Canis lupus*) in China (accession no. JF780946). The also report that none of the *nad1* sequences of *T. cati*, *T. canis* and *T. leonina* from Iran were 100% homologous with reference sequences in GenBank, except for an isolate of *T. canis* which showed 100% homology to an isolate from Australia (accession no. AJ920383). Phylogenetic analysis indicates the differences within Iranian isolates of *T. cati*, and a large difference between isolates of *T. cati* from Iran and three isolates of *T. cati* from China (accession nos. JF780941, JF780942, JF780945 for *cox1* and JF833957 and JF833959 for *pnad1*) (Mikaeili *et al.*, 2015).

In conclusion, the results of present study indicate that PCR method based on sequence of *cox1* and *nad1* genes of mtDNA is a suitable technique for the differentiation of *T. canis and T. leonina* species. Also we demonstrated the sequence variation in mitochondrial genes in *T. canis* and *T. leonina* isolates from Karaj, Iran. Genetic characterisation of these species can be used for future studies of genetic variability and specific identification of *T. canis* and *T. leonina*. The results of the present study and previous studies have indicated that mtDNA regions may be used as genetic markers for the identification and differentiation of *Toxocara* species. Also, genetic variability among *Toxocara* isolates could be revealed by sequences of mtDNA regions. More attention to molecular and bioinformatics studies can be helpful in this regard.

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