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Original Article

Genetic Variation in Mitochondrial *COX1* and Ribosomal *ITS2* Genes of *Toxocara Canis* in Stray Dogs in Zabol, Southeast Iran

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ABSTRACT

Background and Aims: Toxocariasis is a zoonotic parasitic disease with worldwide distribution caused by the larval stage of ascaridoid nematodes of dogs (*Toxocara canis*) and cats (*Toxocara cati*). The study was accomplished to determine the sequence variation in *ITS2* and *COX1* genes within isolates of *Toxocara canis*.

Materials and Methods: Two hundred Stool samples were collected randomly from dogs in public parks and streets from different regions of Zabol. Thirty samples containing eggs were isolated from the feces using Formalin ether 10% and centrifugal flotation. Genomic DNA was extracted, and *COX1* and *ITS2* were amplified by PCR-RFLP and sequenced. Sequence data were aligned using the BioEdit software and BLAST program and compared with published sequences in GenBank. The phylogenetic relationship between isolates of *T. canis* from Zabol city with other regions based on sequences obtained from *COX1* and *ITS2* genes and using MEGA7.0 software was investigated.

Results: For all samples, amplicons of about 388 and 422 base pairs were produced by PCR for *ITS2* and *COX1*, respectively. Drawing the phylogenic tree of *ITS2* and *COX1* sequences of isolates of *T. canis* showed that the identified genotypes are not only different from each other but also from other parts of the world.

Conclusions: Our result showed that genetic variation among isolates of *T. canis* from Zabol is very low. For a deeper understanding of genetic diversity among populations of *Toxocara*, it is recommended to analyze more isolates from various geographical areas and variable genetic markers.

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Introduction

Toxocariasis is a zoonotic parasitic disease with worldwide distribution caused by the larval stage of ascaridoid nematodes containing human and animal species, such as *Toxocara canis (T. canis)*, *T. cati*, *T. vitulorum* and, *T. leonina* [1, 2]. *T. canis* is one of the most widespread public health and economically zoonotic parasitic infections humans share with dogs, cats, and wild canids [3, 4]. Epidemiological surveys have reported that the prevalence of *T. canis* in dogs ranged from 6.3-29% in different parts of Iran [5-7]. The prevalence of human toxocariasis in various parts of Iran ranges from 1.39 to 34.48% [8-13]. Significantly, humans become infected by accidentally ingesting viable eggs from contaminated soil and food. After hatching, emerging larvae are incapable of completing their life cycle and develop into mature adult worms in the human host and cause a range of clinical syndromes such as ocular larva migrans, visceral larva migrans, eosinophilic meningoencephalitis, neurotoxocariasis and covert toxocariasis [1, 2, 14, 15]. Traditionally, Toxocara species are known to be consistent with their morphological characteristics and predilection in a specific host species [16, 17]. Since the morphological identification of some ascaridoid nematodes especially at the larval and egg stages, is challenging [18], thus the analysis of genetic variation is necessary for studying genetic structures and population biology of parasites [19]. Currently, molecular methods such as polymerase chain reaction (PCR) of a selected target restriction fragment length polymorphism (RFLP) using ribosomal and mitochondrial markers have been developed and used widely for the diagnosis and genetic differentiation of *Toxocara* species [20-22]. Genetic variation plays a significant role in the adaptability and survival of a parasite once its environment changes. Accurate analysis of this variation is appropriate for studies on pathogenesis, taxonomy, population biology, and epidemiology of parasites. Various studies have been reported that certain genetic regions, such as the nuclear ribosomal DNA (rDNA) and mitochondrial could certify reliable markers for determining the phylogenetic relationships and genetic variation within and among the Toxocara species [21, 23-25]. The mitochondrial and ribosomal gene sequences have been used to analyze genetic variations of Toxocara in various parts of the world [26-28]. Previous studies are done on the molecular characterization Toxascara species. For the first time, Jacobs et al. (1997) reported the differentiation of nematodes of *T. canis*, *T. cati* and *T. leonina* based on second internal transcribed spacer (*ITS2*) sequences [20]. In respect of the high prevalence of *T. canis* in dogs in the world and shedding Toxocara eggs in the environment, which are transferable to humans, the identification of *T. canis* is substantial for the planning of prevention and control programs in human and animal communities [29]. Despite the high prevalence of *T. canis* in Zabol dogs (27.5%) [13] and their medical importance, there is no information from genetic analysis of those

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parasites from Zabol city. Therefore, this study was designed for the characterization and analysis of genetic variation within isolates of *T. canis* in stray dogs in Zabol by sequencing mitochondrial cytochrome c oxidase subunit 1 *COX1* and second internal transcribed spacer *ITS-2* of nuclear rDNA.

Materials and Methods

Collection of *T. canis* eggs

This cross-sectional study was meted out from June to December 2018 in Zabol, located in Southeast Iran, with 394,029 populations having a hot and dry climate. The average annual precipitation and temperature are 60 mm and 22°C, respectively. The sample volume needed to determine the contamination with *T. canis* was estimated according to the study of Emampour et al. in 2013 [5]. A complete of 200 stool samples were collected randomly from dogs in public parks and streets from different regions (urban and rural) of Zabol; samples were placed in labeled containers and transferred to the parasitology laboratory of Zabol University of Medical Sciences. This research project was reviewed and approved by the Ethics Committee of Zabol University of Medical Sciences, Iran. Project No. IR.ZBMU.REC.1397.056.

Microscopy method

30 samples containing Eggs were isolated from the feces using formalin ether 10% and centrifugal flotation [30], and eggs were preserved in 70% (v/v) ethanol at -20 °C till extraction of genomic DNA.

DNA extraction

Samples were entirely washed in distilled water to make ethanol. Total genomic DNA was extracted using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), with the subsequent modifications: Once the addition of 50 µL lysis buffer, the samples were subjected to three manual sonication cycles (1/5 min each) followed by three freeze-thaw cycles (liquid nitrogen/water bath at 56 °C) and proteinase K digestion was performed overnight. The DNAs were kept at -20 °C till PCR amplification.

PCR amplification

The complete nuclear *ITS2* sequence and sequences of the mitochondrial *COX1* were amplified by PCR using the following primers: *ITS2* (forward: 5′-AGTATGATGGGCGCGCCAAT-3′, reverse: 5′-TAGTTTCTTTTCCTCCGCT-3′); *COX1* (forward: 5′-TTTTTTGGGCATCCTG AGGTTTAT-3′, reverse: 5′-TAAAGAAAGAACATAATGAAAATG-3′) [31, 32]. The PCR amplification was performed in 50 µl reaction volumes containing 25 µl 2× Taq PCR MasterMix (Cinnagen, Iran), 10 µl DNA template, 13 µl deionized distilled water, and 1 µl of each primer (25 pmol/µl; Cinnagen, Iran). The temperature specification was one cycle of 94 °C for 5 min (Initial denaturation), followed by 35 cycles of 94 °C for 30 s for denaturation, 55 °C for 30 s for annealing, and 72 °C for the 30s for extension, and final extension of 72 °C for 5 min. A sample including distilled water instead of template DNA was contained in each run as a negative control. PCR products were separated by electrophoresis on a 1.5% agarose gel in TAE (Tris 0.09 M, Acetic acid 0.09 M, EDTA 0.5 M) at 70V for 1h. Gels were

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stained with 5.3 µl ethidium bromide 10% (Roche, Germany), and the bands were visualized using a UV transilluminator and digitally photographed. *ITS2* and *COX1* PCR products (5 µl) were digested with 0.5 µl restriction endonuclease RsaI (New England Biolabs (NEB), R0167S) for 3h at 37 °C. Restriction fragments of amplicons were electrophoresed using a 3 % (w/v) agarose gel at 70 V for 1 hour, visualized on a UV transilluminator, and digitally photographed [33].

Sequencing and phylogenetic analysis

PCR sequencing was performed to stabilize the results of the PCR-RFLP method. The consensus sequences were compared with one another and GenBank reference sequences using the BioEdit software (http://www.mbio.ncsu.edu/bioedit/bioedit.html) and BLAST program (http://www.ncbi.nlm.nih.gov/) and sequence alignments by MEGA 7.0 software.

Results

For all samples, amplicons of about 388 and 422 base pairs (bp) were produced by PCR for *ITS2* and *COX1*, respectively (Figs. 1, 2). After purification of the PCR product, all samples were sequenced by Takapozist company, and also the sequencing sources for *ITS2* and *COX1* were carefully examined using Chromas software. In this study, results of multiple alignments to compare sequences, identification of variable regions, and the number of transitions and transformations in the studied sequences, multiple alignments were performed exploitation MULTALIN software (http://multalin.toulouse.inra.fr/multalin/multalin.h) software.

T. canis isolates were sequenced and compared with each other and with other parts of the world and subjected to multiple alignments. The phylogenetic relationship of *ITS2* and *COX1* sequences of isolates of *T. canis* from Zabol city using MEGA7.0 software showed that the identified genotypes are the same and there are no different from each other and other parts of the world (Fig. 3). In the present study, exploiting the BLAST program, all 30 isolates from dogs were identified as *T. canis*.

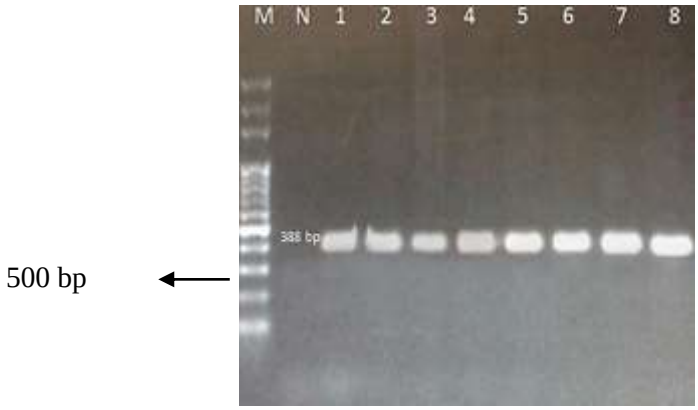

Fig. 1. PCR amplification of *ITS-2* (~388 bp) of *T. canis* from the feces of stray dogs in Zabol on 1.5% agarose gel. M – 100bp DNA ladder, N: negative control

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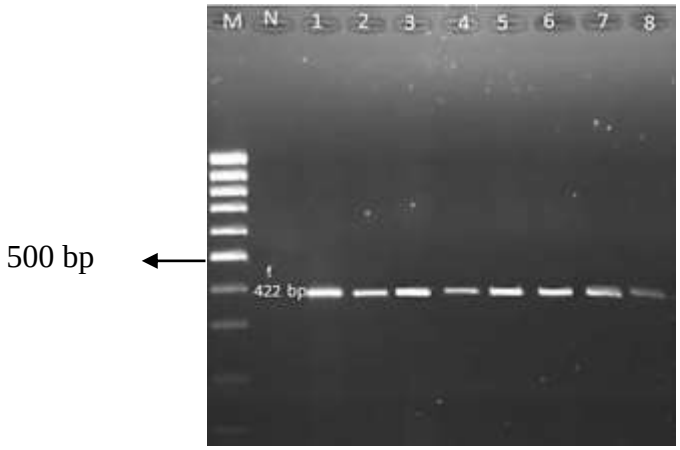

Fig. 2. PCR amplification of *COX1* (422bp) of *T. canis* from the feces of stray dogs in Zabol on 1.5% agarose gel. M – 100bp DNA ladder, N: negative control

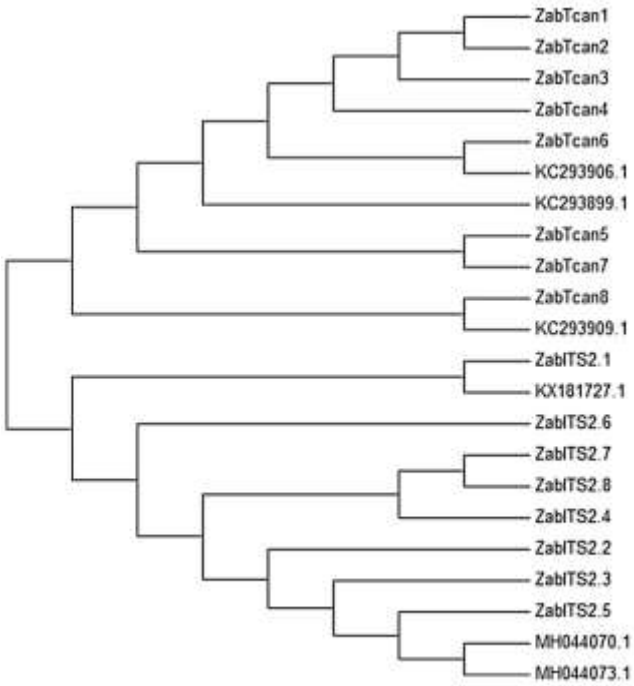

Fig. 3. Phylogenetic relationship of *COX1* and *ITS2* sequences of isolates of *T. canis* from Zabol using MEGA7.0 software

Discussion

The genetic analysis of parasites is an important factor in determining epidemiology and the management of parasitic diseases in humans and animals [34]. *T. canis* is a common parasitic nematode of dogs with public health considerations in dogs and humans [35, 36]. The prevalence of *T. canis* has been reported to be nearly high in Zabol dogs [13]. However, Toxocara's genetic and phylogenetic situation in this region has never been investigated. So as that understand the genetic structure and particular phylogenetic

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position of *T. canis* obtained from dogs in Zabol, genomic DNA was extracted, and *COX1* and *ITS2* genes were amplified. Extending from previous studies, specific PCR techniques are sensitive and specific and provide molecular tools for designation and molecular epidemiological studies of Toxocara infections in humans and animals [20, 37-39]. The PCR-RFLP is a suitable and reliable method for identifying Toxocara species that is based on the digestion of the PCR products by restriction enzymes or endonucleases and an important technique for epidemiological studies, particularly of environmental samples as a result of it permits the differentiation of Toxocara eggs isolated from the soil [20, 33, 40, 41]. Ribosomal genes such as first *ITS-1* and the second *ITS-2* are almost preserved and are typically used as genetic markers within the specific identification of nematodes. Previous studies confirm that *ITS-1* and *ITS-2* genes are suggested for the molecular differentiation of Toxocara species and can enable a reliable tool for identification functions, especially at the larval and egg stages [33, 42, 43]. Our results in this study indicated that the sequence of *ITS2* and *COX1* region of *T. canis* isolates in Zabol city is not only different from each other but also from other parts of the world and genetic diversity within isolates of *T. canis* from Zabol is extremely low. Zhu et al. (2001) found that the level of sequence differences among species ranges from 26 to 50%, whereas there is almost no variation (0-0.6%) within the same species [21]. Also, a comparison of ITS sequences of *T. canis* from Poland with sequences deposited in GenBank showed that the scope of interspecies variability of the species did not exceed 0.4%, while in *T. cati* the differences did not exceed 2%. [42]. The study conducted by Borecka (2004) for the difference between Toxocara spp. eggs isolated from soil by molecular methods showed that *ITS2* PCR products of *T. canis* and *T. cati* were similar in size. Therefore, this PCR approach alone could not differentiate the eggs of *Toxocara* spp. The authors used the PCR-RFLP technique, and this methodology gave the impression to be helpful for the characterization of the species level for *Toxocara* spp. [41]. In addition to nuclear *ITS-1* and *ITS-2* rDNA sequences, recent studies have shown that mitochondrial DNA such as the mitochondrial cytochrome c oxidase subunit (*COX1*) gene is a helpful genetic marker for the identification of ascaridoid nematodes and also identify parasites such as *Ancylostoma ceylanicum, Spirometra spp., Echinococcus granulosus* [25, 44-51]. Mitochondrial DNA has several benefits when studying investigating population genetic structures, and phylogeny of parasitic nematodes due to their higher mutation rates than nuclear genes and preserved genome structures. Mitochondrial DNA has necessary implications for various fundamental areas, as well as biochemistry, biology, and physiology [24, 52, 53]. Mikaeli et al. (2015) showed the diversity of COX1 and NAD1 mitochondrial gene sequences in dog and cat nematodes in several regions of Iran. Sequence and phylogenetic analysis of *COX1* and NAD1 genes showed significant genetic diversity in

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and between 9 isolates of T. canis, 32 isolates of *T. cati*, and 19 isolates of *Toxascaris leonina* and these genes can be used to study the genetic variation of ascaridoid nematodes [54]. This study is the first phylogenetic analysis of *T. canis* from dog Zabol city, Southeast Iran.

Conclusion

This study incontestable the existence of genetic diversity in mitochondrial genes and ribosomal genes among isolates of *T. Canis* in Zabol, southeast Iran. In general, we conclude that the sequence of *ITS2* and *COX1* region of *T. canis* isolates in Zabol city differs not only from each other but also from other parts of the world. The *ITS2* can be utilized for particular identification of Toxocara nematodes and specific primer design, although the *COX1* might provide valuable data for future phylogenetic and diversity genetics studies on the Toxocara nematodes. For a deeper understanding of genetic diversity among populations of Toxocara, it is recommended to analyze more isolates from various geographical areas and variable genetic markers.

Conflict of Interest

The authors declare that there are no competing interests.

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