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Original Article

Molecular Identification of Nematodes (Superfamily: Strongylida) Traced in Herbivores Excrement Found in Wildlife from Western Iran

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Abstract

Background: In wildlife, the identification of parasitic infections should be pursued seriously in countries facing endangered species of animals in their geographical territories. We aimed to increase understanding of the possible role of wildlife herbivore reservoirs in the emergence of helminth infections in Kermanshah Province, western Iran.

Methods: Sixty-five feces from Gazelle (*Gazella subgutturosa*) (N = 36 samples) and wild goat (*Capra aegagrus*) (N = 14 samples) were investigated. The samples were microscopically examined for gastrointestinal helminth eggs, and genomic DNA was extracted from the identified eggs. The internal transcribed spacer 2 (*ITS2*) region of ribosomal DNA was amplified and sequenced. Phylogenetic analysis of the nucleotide sequences confirmed the species identity.

Results: The most common species circulating in the hosts were *Teladorsagia circumcincta*, *Marshallagia* spp., and *Nematodirus oiratianus*, all of which are reported in the wildlife in western Iran for the first time.

Conclusion: These findings emphasize the importance of continuously assessing the parasite status of wildlife and similar routine surveillance in domestic environments to detect and manage potential zoonotic parasite species.

Introduction

Parasitic infections due to nematodes are among the health-threatening biological factors in the natural environment. These pathogenic helminths may influence the physiology, behavior, and reproduction output of animals in the natural environ-

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ment, specifically regarding endangered species (1, 2). It is also known that environmental changes like climate factors can enhance the vulnerability to nematode infection and the intensity of parasitosis in wildlife (3). Besides, some species of nematodes in wild animals can occasionally spread to human populations and their domestic animals, exhibiting as emerging new pathogenic agents (4).

The main clinical symptoms of nematode infections in livestock are exhibited by the load of certain parasites in their infected hosts (5). They strongly affect their general health status, population dynamics, and susceptibility to acquiring other non-parasitic diseases (6, 7). Although wild ruminants are generally known to be more resistant to parasitic helminths than domestic ones, it should be taken into account that these biological agents remain a significant concern for endangered animals (8, 9). So, investigating parasitic infections and their genetic studies in wildlife will increase our understanding of the parasite ecology and the cryptic species that are morphologically similar (10, 11). Regarding appropriate climatological conditions and the natural ecosystem of western Iran for the wild herbivores, our present knowledge concerning the parasite status in this region is scarce (12). The most prevalent gastrointestinal nematode (GIN) fauna in ruminant livestock includes *Haemonchus, Cooperia, Trichostrongylus, Ostertagia, Nematodirus, and Oesophagostomum* species.

Wild cervids, including deer, caribou, elk, and pronghorn, are ruminants that can contract GIN infections by grazing on pastures contaminated with infective larvae (13). Therefore, the transmission of GIN between domestic and wild ruminants can interfere with our efforts to control these infections in domestic livestock. Moreover, in the same way, the wild ruminants may be parasitized by their nearby rural environment's domestic grazing cattle, sheep, and goats (14). For instance, Hrabok et al. had previously highlighted a connection between the parasite communities in domestic livestock and reindeer. Their findings demonstrated that reindeer could act as susceptible hosts for significant GIN species affecting sheep (*Teladorsagia circumcincta* and *Haemonchus contortus*) and cattle (*Ostertagia ostertagi* and *Trichostrongylus axei*) (15). Through experiments on animals, McGhee et al. demonstrated that *H. contortus* can be transmitted between deer and domestic livestock. They identified white-tailed deer as significant hosts for these Haemonchus in the southeastern region of North America (16).

Epidemiological studies have shown that trichostrongylosis is a common parasitic infection among domestic ruminants and humans in various regions of Iran (17). The prevalence of total infections with the nematodes was 38.9%, 74.6%, and 84.6% among cattle, sheep, and goats, respectively. Eleven species of trichostrongylid nematodes, including *H.contortus*, *Marshallagia marshalli*, *T. axei*, *T. colubriformis*, *T. vitrinus*, *Ostertagia trifurcata*, *T. circumcincta*, *M. occidentalis*, *O. lyrata*, *O. ostertagi*, and *Cooperia punctate* were recovered from the ruminants. In Iran, moreover, the most prevalent trichostrongyloid nematodes in cattle, sheep, and goats were *O. ostertagi* (26.4%), *M. marshalli* (64.4%), and *T. circumcincta* (69.2%), respectively (18). *T. circumcincta*, initially classified in the genus Ostertagia, is a parasitic nematode in the stomach. It infects sheep and goats globally, leading to weight loss, decreased wool production, and even death (19).

The prevalence rates of 17.3 to 47.2% for *T. circumcincta* have been reported in sheep and goats in different provinces of Iran (20). Detection of characteristic eggs in stool samples of humans and animals is a routine diagnostic method for Strongylida infections (21). Recently, PCR techniques have been employed for accurate identification and phylogenetic analysis of nematodes (22, 23). In the present study, internal transcribed spacer 2 (*ITS2*) region of ribosomal DNA was used for characterization and phylogenetic analysis of wild ruminant parasites using their fecal materials to know the possible role of wild animals in

the emergence of zoonotic infections in western Iran.

Materials and Methods

Study area

The study was carried out in Kermanshah Province, western Iran (34.3277° N, 47.0778° E, and 1350m above sea level), areas including Ghraviz Wildlife Sanctuary (41760ha) and Zelzard (80000ha). Kermanshah is bordered by Iraq to the west and is located beside two cities of Iraq, Soleimaniye and Diyali. Kermanshah is located between two cold and warm regions and experiences a moderate and mountainous climate. It rains most in winter and is moderately warm in summer. The annual rainfall is 500 mm. The average temperature is 15.6 °C in spring, 25.3 °C in summer, 11.3 °C in autumn, and 3.3 °C in winter. Due to the climatological situation and technical preferences of the Department of Environment in Iran, some endangered animal species, like Persian Fallow Deer, have been temporarily transferred to nearby provinces. Consequently, the samples of Persian Fallow Deers of western Iran were provided from Karkheh and Dez protected area in Khuzestan, located in the southwest area of the country near the borders with Iraq and the Persian Gulf (31.4360° N, 49.0413° E, 12 m above sea level). This province is known to have hot weather with about 50 °C and 9 °C in July and March, and a landscape of rolling hills, mountainous regions, and marshlands. The three large rivers of Iran, Karoun, Karkheh, and Maroun, make the Khuzestan plain especially suited for agriculture (24).

Sample collection

The left feces in the wild environment were obtained two times, from October 2022 to Jun 2023: dry season in October 2022 (autumn, reproductive) and wet season in Jun 2023 (spring, rainfall). The collected feces were precisely sampled within 400 m-3 km intervals during the day. The samples were labeled, dated, placed into a sterile plastic bag, and transferred to the laboratory. Sixty-five samples were collected from Gazelle (*Gazella subgutturosa*) (N = 36 samples), wild goat (*Capra aegagrus*) (N = 14 samples) from Kermanshah province, and the Persian fallow deer (*Dama dama Mesopotamia*) (N = 15 samples) from Khuzestan province. Five grams of each fecal sample were pre-served as homogenized feces in 10% formalin and 70% ethanol for microscopic and molecular methods, respectively.

Parasitological methods

Fecal samples were examined for the presence of helminth eggs and larvae using the formalin-ether sedimentation technique. Three slides were prepared for each sample. Slides were microscopically screened at 100x, 200x, 400x, and 1000x magnification, and eggs were identified based on characteristic shape and parasitic stage (25).

Molecular techniques

Due to the possible mixed infection of Strongylida in the wild herbivores and similar morphological characteristics amongst these gastrointestinal nematodes, aiming for molecular confirmation, DNA extraction was carried out for the eggs collected from feces in similar morphology groups independently. The genomic DNA of each sample was extracted by FavorPrep™ Stool DNA Isolation Kit (Favorgen, Taiwan) according to the manufacturer's instructions and stored at -20 °C until the performance of PCR amplification. A polymerase chain reaction (PCR) specific for the ribosomal DNA internal transcribed spacer 2 (*ITS2*) region was carried out with forward primer NC1: 5-ACGTCTGGTTCAGGGTTGTT-3 and reverse primer NC2: 5-TTAGTTTCTTTTCCTCCGCT-3.(26). PCR was conducted in a 25 µL reaction mix under standard cycling conditions with proper positive/negative controls; products were checked on agarose gel and subsequently sequenced bidirectionally using Sanger sequencing.

Analysis of sequence and population genetics

The quality of crude sequences was improved using the Chromas 2.6.6 program by removing areas with poor quality at both ends of the sequences. The consensus of confident sequences was examined using the NCBI (nucleotide collection) database. Multiple DNA sequences were aligned using the ClustalW program (http://www.ebi.ac.uk/clustalw/) and afterward trimmed. Finally, a 320 bp consensus sequence length was used for phylogenetic analysis. A maximum likelihood tree was constructed based on the Kimura 2-parameter model, and pairwise comparisons were made of sequence differences within and among species using the MEGA 11 software. The topology of the phylogenetic tree was evaluated using the bootstrap test based on 1000 replications. For phylogenetic analysis of *T. circumcincta*, we used 28 sequences, including 19 *ITS2* sequences of *T. circumcincta* available in GenBank, six sequences from the current study, as well as those of *M. marshalli* (accession no. HQ389231), *T. colubriformis* (accession no. HQ389232), and outgroup *Necator americanus* (accession no. Y11734). The phylogenetic tree of *N. oiratianus* was constructed based on the Tamura 3-parameter model. Twenty-three sequences were used, including 7 *ITS2* sequences of *N. oiratianus* available in GenBank, one sequence from the current study, as well as those of *N. helvetianus, N. abnormalis, N. andersoni, N. tarandi, N. battus, N. filicolli, N. spathiger, Nematodirella longissimespiculata,* and outgroup *Necator americanus* (accession no. Y11734).

Results

Parasitological findings

A total of 65 fecal samples from 3 wild ruminant species were randomly collected and analyzed to find any parasitic eggs. Of 65 samples, 12 (18 %) were positive microscopically (Table 1), of 36 samples of gazelle, 10 (27%) were positive for Strongylida. Of 14 wild goat samples, 2 (14%) were positive. No infections were tracked in Persian fallow deer feces (Table 1, Fig. 1).

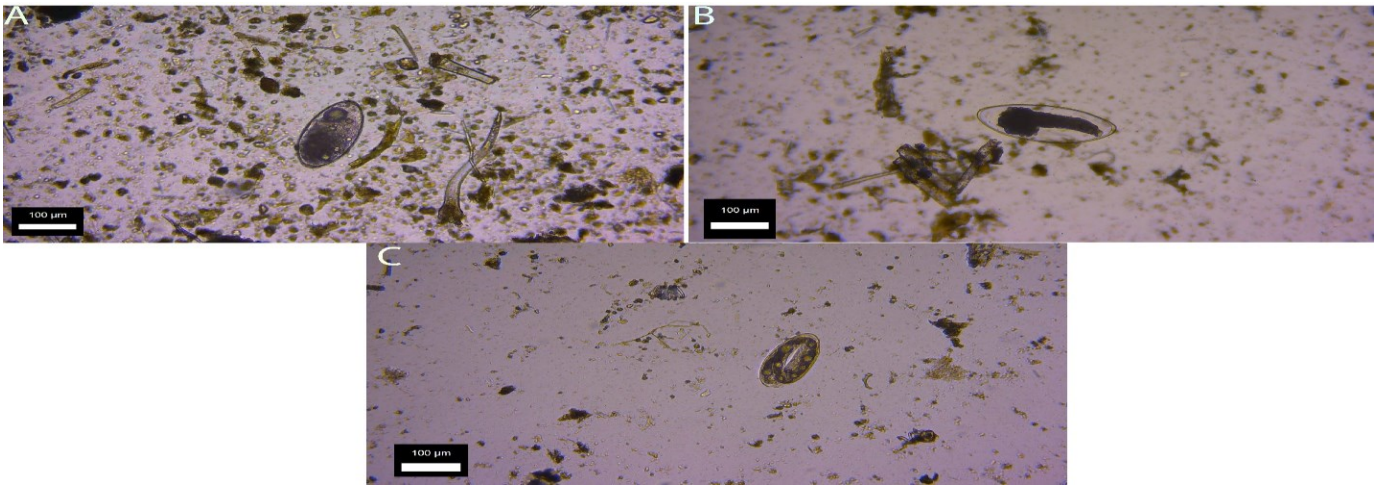

Fig. 1: Light microscope photographs of Strongylida eggs identified in fecal samples from wild ruminants in the west of Iran. They molecular methods identified them as A: *T. circumcincta*; B: *N. oiratianus*, C: *Marshallagia.* spp, Scale bar 50 µm (Original)

The molecular confirmation of parasites

The *ITS2* region of Strongylida was amplified to identify the species in all 12 samples, which were positive by the parasitological method. Amplicons were visualized by electrophoresis on a 1.5% agarose gel, producing a single amplification of a 328 bp band. After sequencing and BLAST analysis, the genus and species were identified in 12 eggs of Strongylida isolates. The sequences were deposited in GenBank, including five *T. circumcincta* (accession numbers: PQ763999, PQ764006, PQ764007, PQ764008, PQ764011) and five *Marshallagia* spp. (accession numbers: PQ807235-39) in *Gazella subgutturosa* and one *N. oiratianus* (accession numbers: PQ764722) and one *T. circumcincta* (PQ763997) in *Capra aegagrus*.

Five of 35 Gazelle feces samples were infected with *T. circumcincta* and five with *Marshallagia* spp. Of the 14 wild goat samples, one *T. circumcincta* and one *N. oiratianus* were characterized. *ITS2* was not seemingly discriminative in distinguishing species of *Marshallagia*.

Table 1: The parasitological and molecular findings of helminths in the herbivores

Animal species (No)	Infection percent	Positive No.	Microscopical results	Helminth species (PCR)
Gazelle) *Gazella subgutturosa*) (36)	10/36 (27 %)	5	Strongylida	*T. circumcincta*
		5	Strongylida	*Marshallagia*.spp
Wild goat (*Capra aegagrus*) (14)	2/14 (14 %)	1	Strongylida	*T. circumcincta*
		1	Strongylida	*N.oiratianus*
Persian Fallow Deer (*Dama dama Mesopotamia*) (15)	0%	0	0	0
Total, 65	12/65 (18%)	12	12	12

Phylogenetic analysis

The sequences were compared with different sequences available in the GenBank database using the BLAST system. The phylogenetic tree was constructed after trimming and comparing the sequences in the GenBank database. The phylogenetic analysis showed that each species was clearly distinguished and placed together with the same species in the GenBank database (Figs 2 and 3). The phylogenetic trees showed that *Teladorsagia* at first diverged from *Marshallagia* and *Trichostrongylus*. Based on the host, *Teladorsagia* sequences in Iran and the GenBank were isolated from ruminants. Phylogenetic analysis based on 28 *ITS2* sequences for *T. circumcincta* (six from this study and others among the available sequences of GenBank) demonstrated that *T. circumcincta* stands alongside other species worldwide (Fig. 2).

The sequences obtained in this study for *N. oiratianus* were compared with those in the GenBank database using BLAST analysis. The results revealed 100% similarity with *N. oiratianus* isolated in Canada (OP879515.1) and 92% similarity with *N. oiratianus* isolated in China (MT193658.1). A maximum-likelihood phylogenetic tree was generated from the final alignment of the sequences. It showed three main clades of *Nematodirus* infecting a variety of host ruminants (cattle, sheep, and camels) and wild ruminants (wild goat).

Fig. 2: Phylogenetic tree of the *ITS2* nucleotide sequences of *T. circumcincta* isolates in this study (▲), and others retrieved from GenBank. A maximum likelihood tree was constructed with MEGA 11 based on the Kimura 2-parameter (K2P) model with *Necator americanus* as outgroup. Bootstrap values are shown at the nodes based on 1000 replicates. The scale bar indicates genetic distance. All the sequences of *T. circumcincta* from GenBank are displayed with their accession number, the host origins, and the original countries

The first clade contained the Iran sequences and one sequence that this study of *N. oiratianus* identified. The comparison revealed variations among and within geographic regions. The phylogenetic tree topology showed similarities and differences among the globally registered sequences of *N. oiratianus* with other species of Nematodirus and our registered sequences worldwide (Fig. 3).

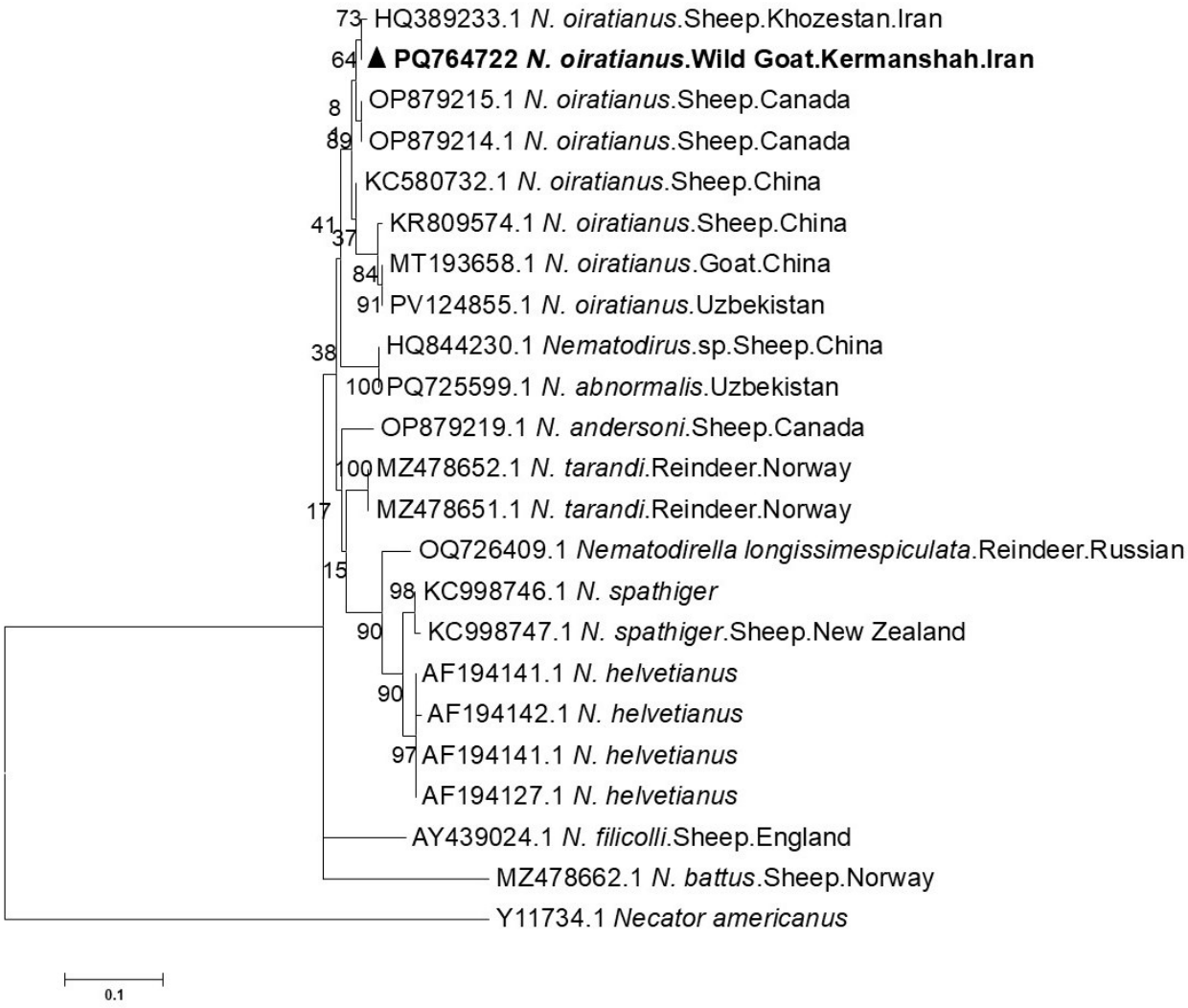

Fig. 3: Phylogenetic tree of the *ITS2* nucleotide sequences of *N. oiratianus* isolate obtained in this study (▲), and other sequences retrieved from GenBank. The maximum likelihood tree was generated with the Tamura 3-parameter model in MEGA 11. Bootstrap values are shown at the nodes based on 1000 replicates. *Necator americanus* is used as an outgroup

Discussion

Investigation of parasites harbored by wildlife animals provides new insight into the status of parasitic infections in natural ecosystems, which can assist programmers in developing effective prevention strategies (27). Wildlife parasitology can lead us to find the origin of recently emerged parasites in domestic animals from the wild territory and vice versa. It is, however, widely assumed that pathogen transmission at the livestock-wildlife boundaries seems bidirectional, involving the circulation of pathogens between like the study in Limpopo province, South Africa that showed a significant overlap of parasite spe-

cies (28). Environmental intervention in parasite transmission can be witnessed in different zoogeographical regions, like what has been recorded in the new regions of the high Arctic and Himalayan highlands regarding the climate change and the increas in vectors (29).

In this study, the Strongylida species including *Cooperia* spp., *Haemonchus* spp., *Oesophagostomum* spp., and *C. tenuicollis* have been identified. Since some of these helminths are of zoonotic species, the present findings may also highlight the potential occurrence of such parasites in human populations. The present study was aimed to investigate the possible existence of gastrointestinal helminths (GIN) in wild ruminants in Kermanshah, western Iran, bordering Iraq, where the lack of reliable information is obviously witnessed. The similar findings in Iraq are not well informative while the helminth profiles in ruminants in the border illustrate cross-border parasite circulation requiring more collaborative surveillance in the region.

In this research, the helminth infection rate in the wild herbivores was seen at eighteen percent that could be related to environmental conditions as well as unpredictable climate changes, facilitating the parasite establishment the region (30) with exception of Persian fallow deer (*Dama dama mesopotamica*) that were not found infected. The negative GIN results of Persian fallow deer (*Dama dama mesopotamica*) can be attributed to the limited home range of this wild herbivore that cannot be persisted forever (31). The present identified species have also been reported in the wildlife from various parts of the world. *T. circumcincta*, *Marshallagia* spp, and *N.oiratianus* are also known to be prevalent in domestic herbivores in Iran from different geographical regions by degrees (32). Similar findings indicate the occurrence of these parasites in the wildlife of Iran (33).

Marshallagia spp. is reported for the first time in gazelles from wildlife in western Iran. *M. marshalli* of this genus was also detected in other domestic and wild ruminants, like in Addax and Dorcas gazelles, in Bouhedma and Orbata National Parks of Tunisia (34, 35) (57.1%), which has also been identified as the dominant GIN species in sheep from Isfahan, central Iran. Moreover, to this helminth, *T. circumcincta* were found in sheep and goats from Qazvin province in northwest Iran (36). Concerning the Nematodirus species, the occurrence of this Strongylida worm in gazelles (22) is documented in an earlier study, while in present research, it was not detected in the gazelles' excrements. Meanwhile in the humid zone of northwest Tunisia, the existence of *N. spathiger* and *N. filicollis* was confirmed (35), and this is worth to mention that the other species *N. oiratianus* was detected in this study in the goats in western Iran. *T. circumcincta*, was the other strongylid detected in gazelles that has also been reported as a prevalent gastrointestinal nematode in herbivores across Iran. It was found in 47.2% of goats in central (36) and in sheep in western Iran (37). Moreover, this gastrointestinal nematode has been known as the most prevalent helminth species in sheep (38) and was also reported in 38% of sheep in Mazandaran province by Naem et al (39). However, from the zoonotical points of views, *T. circumcincta* has been reported in humans in Azerbaijan and northern Iran (39), which highlights the health importance of GIN in regions where infections with these helminths are prevalent among domestic herbivores as well (40). Revealing of *T. circumcincta* in both gazelles and wild goats along with its documented records in humans in Iran (23), underscores the need for epidemiological studies to transmission dynamics at the wildlife-livestock and human interface.

It is noteworthy that other zoonotic GIN worms, such as Haemonchus and Paramphistomum, have been also reported with a high prevalence rate of 39.1% amongst domestic animals in Andhra Pradesh state of India (41). However, review of the literature shows similar close studies with that occasionally was performed in the wildlife (42). Phylogenetic trees were constructed separately for *T. circumcincta* and *N. oiratianus*, showing few differ-

ences between the isolates obtained in this study and those previously reported worldwide. The phylogenetic tree represented that *T. circumcincta* specimens obtained in the current study and other isolates from sheep and goat in Iran were placed in the same clade. Besides, the sequences of *T. circumcincta* from gazelle and wild goats showed high (close) similarity. The phylogenetic analysis also indicated that *N. oiratianus* specimens obtained in this study were grouped with a sheep isolate from Khuzestan (HQ389233), demonstrated close genetic homology. This study was mainly decided to increase understanding of possible share of wildlife reservoirs towards the emergence of zoonotic helminth parasites to domestic animals and humans. The presented results from Kermanshah region bordering Iraq provides a meaningful descriptive epidemiological insight into the natural circulation of helminth infection in the wildlife of western Iran. Aiming to control the increasing risk of helminth gastrointestinal infections in the wildlife in western Iran a precise continual monitoring programs in the area with special focus on gazelles and wild goats as the key reservoirs is highly recommended. Referring to invaluable approach to "One Health" deworming measures from veterinarians' side in livestock along with limiting share grazing besides trying to treat the wild animals could be a scientific program action in this regard.

One of the basic limitations faced by the researchers in the present study was the sample size, which could not be increased statistically to obtain more desired results. Relying on fecal sampling left by animals in their own habitats is an ethical, noninvasive approach to determine the parasitological status in wildlife. Meanwhile, through this selected approach, the researchers are certainly encountering challenges related to sample size, as well as difficulties in tracking results among hosts with dispersed populations (42).

Conclusion

The present study supports a significant hypothesis that wild ruminants may host parasites, including gastrointestinal nematodes and zoonotic species, in their natural environments. *T. circumcincta* and *Marshallagia* spp. were probably the most common species documented for the first time in the wildlife of western Iran.

These results highlight the urgent need for ongoing surveillance of parasitic infections in wildlife and their surrounding domestic environments. Such bilateral scientific plans can practically predict the possible causes of emerging and reemerging infections in different countries.

Ethics statement

This study protocol was approved by the Ethics Committee of Tehran University of Medical Sciences, Iran (Ref. No. IR.TUMS.SPH.REC.1402.082).

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Conflict of Interests

The authors declare no conflict of interest.

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