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Identification of *Trichinella* species among wild carnivores in north, northeast, and northwest provinces of Iran

Mohammad Reza Khoshvaght, Elahe Ebrahimzadeh, Nooshinmehr Soleymani, Moein Abolhasani Darounkola, Hassan Borji*

Department of Pathobiology, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran.

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Abstract

Trichinella britovi is a species circulating in Iran and has already been observed in wild and domestic carnivores. The present study aims to determine the occurrence of *Trichinella* species in Iran's most important provinces, including the northern, northeastern, and northwest regions. In total, 93 carcasses of golden jackals, collected due to road accidents between 2019 and 2022, have been obtained from Ardabil, Mazandaran, Golestan, North Khorasan, and Khorasan Razavi provinces, Iran. Genomic DNA (four positives out of 93 carcasses) from each Trichinella genotype was amplified in a multiplex polymerase chain reaction (PCR) reaction. To confirm the multiplex PCR results, the sequences of the PCR products with cytochrome oxidase subunit I were sent to a reference laboratory within the European Union. Bioinformatics software was used to verify the sequencing results of primer fragments and to compare and analyze the results obtained with the sequences stored in the NCBI genetic database. Following multiplex PCR amplification, a two-band pattern was observed. *Trichinella britovi* larvae displayed the expected band pattern of 127 bp and 253 bp. The PCR products amplified using primers specific for the *cytochrome oxidase subunit I* gene showed 861 base pair bands in all four samples analyzed. The obtained sequences of cytochrome oxidase subunit I from all four individual muscle larvae had 100% identical haplotypes except for one nucleotide transversion in one sequence. As a result of this study, valuable insights into *T. britovi* prevalence and phylogenetic distribution among Iranian golden jackals have been gained in terms of prevalence and phylogenic distribution.

Introduction

Trichinellosis is a cosmopolitan food-borne parasitic zoonosis caused by various species in the genus *Trichinella*.[1,2] This nematode poses a significant health risk to humans and is transmitted through the consumption of meat or meat products containing the parasite.[3] Carnivores and domesticated and wild swine species are the main natural reservoir hosts for these parasites [4] Raw or undercooked pork meat is a common source of human infection, but raw meat and products from other animals such as wild boar, bears, cougars, foxes, badgers, jackals, walruses, dogs, and even herbivores like horses can also be sources of infection.[5,6]

Until recently, 12 taxa of *Trichinella* have been described in animals and humans, including the encapsulating species *Trichinella murrelli, Trichinella nelsoni, Trichinella spiralis, Trichinella nativa, Trichinella britovi, Trichinella patagoniensis, Trichinella chanchalensis* and genotypes *Trichinella T6*, *T8,* and *T9* exclusive to mammals, and nonencapsulated species *Trichinella pseudospiralis, Trichinella zimbabwensis* and *Trichinella papuae* infecting mammals and birds or mammals and reptiles.[7-10] Identifying specific *Trichinella* species is crucial on determining the possible source of infection, the parasite's geographic origin, and the risks of infection for domestic animals and humans due to this disease.[11] In the 19th century, the sylvatic cycle of *Trichinella* spp. began to evolve. Based on epidemiological data on *Trichinella* species, approximately 95 countries on all continents continue to report infections in animals/and/or humans, including the domestic cycle (in 32 countries; 16.30%), the wild cycle (in 75 countries; 38.30%), and the human cycle (in 47 countries; 23.90%).[12]

***Correspondence:**

Hassan Borji. DVM, PhD
Department of Pathobiology, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran
E-mail: hborji@um.ac.ir

Trichinella spiralis was detected in minks (6.92%) and synanthropic rats (1.96%).[13] Despite this, our epidemiological knowledge is still lacking in several countries and dates back a century or more. *Trichinella britovi* has already been observed in wild and domestic carnivores in Iran.[14-17] However, epidemiological data regarding other taxa of the *Trichinella* genus is still lacking in Iran. Infections in wild carnivores have been reported. An old report mentioned *T. spiralis* in the southern regions of Iran, but this species has not been confirmed with molecular analysis. Thus, the present study aims to determine the occurrence of *Trichinella* species in the region. There have been reports of infection in golden jackals (*Canis aureus*) in Iran's north, northwest, and northeast. A thorough understanding of the epidemiology of *Trichinella* species is essential in developing of a control program to reduce the transmission of the parasite cost-effectively.

Materials and Methods

Regions of study. We have selected three diverse regions for this research based on their climate conditions and geographical characteristics. The northern, northwest, and northeastern provinces of Iran have been chosen for the study. While each region in the country retains its unique features, they also experience different climates. There seems to be a difference in the genetic makeup of carnivores infected with *Trichinella* and the rate at which they contract the infection in different environments. The northern region of Iran, located along the Caspian Sea at 36°23′N 52°11′E (Mazandaran) and 37°20′N 55°09′E (Golestan), experience a humid and warm climate. This region is characterized by dense forests and abundant vegetation making it an ideal habitat for various wildlife species, such as pigs, boars, jackals, and foxes. The presence of these carnivores in the northern region, highlights the importance of studying the dynamics of Trichinella infection in this area. To the northwestern region of Iran, bordered by Türkiye and Armenia border at 38.2514° N, 48.2973° E, consists of mountains, plateaus, and valleys, with varying climates ranging from semi-arid to arid. This region was chosen due to the presence of common strains shared between neighboring countries and Iran, aiming to assess the movement of strains in the environment. The northeastern region of Iran, near the borders of Afghanistan, Turkmenistan, and Pakistan, features vast deserts, plateaus, and mountain chains. The climate in this region is primarily semi-arid and arid, with unique desert and semi-desert ecosystems characteristic of this type of climate.

Sampling. For this study, five provinces of Iran were sampled, namely Ardabil (northwest), Mazandaran, Golestan (north), North Khorasan (37°22′N 57°16′E), and Khorasan Razavi (36° 15′ N and 59° 28′ E, northeast; Fig. 1). In total, 93 carcasses of golden jackals were found in the mentioned areas as a result of road accidents between 2019 and 2022, which was the sample size for the study. In particular, there were 28 carcasses from the northeastern region, 35 from the northern region, and 30 from the northwestern region. Over 10.00 g of muscle tissue were trimmed from each animal's tongue, diaphragm, and forearms. As soon as the samples were transported to the laboratory, they were preserved in ice and examined as soon as possible after collection. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed, as outline by the Ferdowsi University of Mashhad, Mashhad, Iran, Ethical Committee (IR.UM.REC.1401.020).

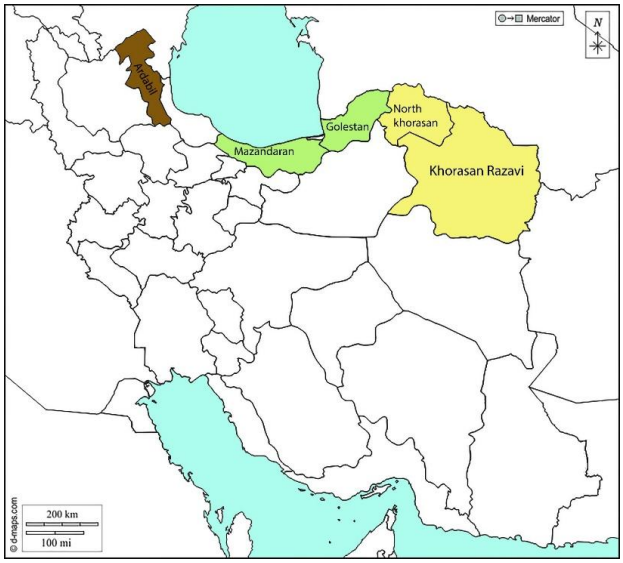

Fig. 1. Map of Iran showing the five provinces that were sampled. Ardabil (northwest) in brown, Mazandaran, and Golestan (north) in green, and North Khorasan, and Khorasan Razavi (northeast) in yellow.

Direct detection of *Trichinella* larvae. According to standard protocol, artificial digestion techniques were used in our laboratory research. Once the muscle tissues were digested, the sediment was washed several times. The sediment from the last washing was examined for larvae with a dissection microscope before being discarded. It was also possible to repeat the procedure using individual samples when the sediment contained *Trichinella* larvae.

DNA extraction. Using a commercial DNA extraction kit (MBST, Tehran, Iran), DNA was obtained from larvae recovered by artificial tissue digestion. Using a Nano Drop spectrophotometer (BioTek Epoch, Winooski, USA), the DNA was quantified after being extracted from the cells.

Multiplex polymerase chain reaction (PCR). The *Trichinella* larvae were identified by multiplex PCR analysis following the protocol described by Pozio, and

Zarlenga *et al*.[18,19] There were five primer pairs used in the multiplex PCR (Table 1), carried out in a total volume of 25.00 µL, using 100 ng of template DNA, 1.00 X reaction buffer (MBST, Tehran, Iran), 200 dNTPs (MBST), 0.50 M of each forward and reverse primer, and 0.50 U of Taq polymerase (MBST). To optimize results, the following cycling conditions were used: denaturation at 94.00 ˚C for 5 min, followed by 30 cycles of denaturation at 94.00 ˚C for 30 sec, annealing at 55.00 ˚C for 45 sec, and extension at 72.00 ˚C for 30 sec. The temperature was raised to 72.00 ˚C for 7 min during the final extension. The PCR-amplified fragments were visualized by agarose gel electrophoresis using 2.00% standard agarose gel. The size of the PCR products was estimated by comparing them to a DNA ladder to estimate the number of *Trichinella* spp. present. To make comparisons, larvae from one reference strain were used for each taxon: *T. spiralis, T. pseudospiralis, T. britovi,* and *T. nelsoni.*

Polymerase chain reaction with cytochrome oxidase subunit I (COX1) and sequencing. By using the forward primer 5′-TACCTATACTACTAAGAGGATTCGGA-3′ and the reverse primer 5′-CTAGTACTCATAGTATGGCTG GTG-3′, amplification of the *COX1* gene was achieved.[20] The PCR was performed in a 50.00 µL volume containing 4.00 µL of extracted DNA, 30.00 µL of the GoTaq G2 Master Mix (Promega, Madison, USA), and 0.50 µL of each primer (10.00 mM). As a general guideline for PCR conditions, the following steps were used: denaturation at 95.00 ˚C for 5 min, followed by 40 cycles of denaturation at 94.00 ˚C for 40 sec, annealing at 57.00 ˚C for 45 sec, extension at 72.00 ˚C for 50 sec, and elongation at 72.00 ˚C for 10 min. The PCR products were electrophoresed on a 2.00% agarose gel stained with Safe dye and separated by electrophoresis on a 2.00% agarose gel. The PCR products were purified using ExoSAP (Affymetrix, Altrincham, UK) according to the manufacturer's instructions before being sent for sequencing to Genomed-Sequence Company, according to their procedure. To determine the sequence of the PCR products, they were sent to a reference laboratory within the European Union for analysis. To confirm the sequencing results of the primer fragments, bioinformatics software was used to verify and compare the results with sequences stored in the NCBI genetic database.

Phylogenetic analysis. Sequences were aligned and edited manually using Clustal Omega. Phylogenetic analysis was performed with MEGA Software (version 11.0; Biodesign Institute, Tempe, USA), applying the Neighbor-Joining method with bootstrap analysis (1,000 replicates). The analysis included the phylogenetic tree of the *T. britovi COX1* gene retrieved from north and northwestern Iran (in this study) and reference sequences deposited in GenBank® related to the *T. britovi* genotypes and other *Trichinella* species. The neighbor-joining method with bootstrapping analysis was applied using MEGA Software (500 replicates).

Results

Prevalence. Four out of 93 (4.30%) golden jackals were found to be infected with *Trichinella* species. In the northwestern region of Iran (Ardabil province), one out of 30 golden jackals (3.33%) were infected with *Trichinella* spp. The rate of infection among golden jackals in the northern region (Mazandaran and Golestan provinces) was two out of 35 (5.71%) compared to one out of 28 (3.57%) in the northeastern region. In the northeastern region, (Khorasan Razavi and North Khorasan provinces), no infections with this parasite were detected.

Multiplex-PCR. Following multiplex PCR amplification, a two-band pattern of 127 bp and 253 bp was observed meaning the *T. britovi* pattern (Fig. 2).

Polymerase chain reaction COX1 and sequencing. The PCR products amplified using primers specific for the *COX1* gene showed 861 base pair bands in all four samples analyzed. The sequence analysis results were analyzed using the MEGA 7 software package, revealing a similarity between the nucleotide sequences obtained from the *COX1* gene and those in GenBank®. Two sequences were derived from the *COX1* gene of samples collected from the north and northwest regions of Iran, and they have been submitted as OR345478 and OR345479. In a multiple alignment analysis of the COX1 sequence, a nucleotide variation was noticed in the isolates from the north of Iran. At position 95 of the nucleotide sequence, there was a transition (T nucleotide instead of C nucleotide), but this did not affect the translation of the amino acid.

Table 1. The five primer pairs used in the multiplex polymerase chain reaction in this study.

Primer pairs	Location of DNA	Sequences
I	ESV	F: 5'-GTTCCATGTGAACAGCAGT-3 R: 5'- CGAAAACATACGACAACTGC-3'
II	ITS1	F: 5'-GCTACATCCTTTTGATCTGTT-3' R: 5'-AGACACAATATCAACCACAGTACA-3'
III	ITS1	F: 5'-GCGGAAGGATCATTATCGTGTA-3' R: 5'-TGGATTACAAAGAAAACCATCACT-3'
IV	ITS2	F: 5'-GTGAGCGTAATAAAGGTGCAG-3' R: 5'-TTCATCACACATCTTCCACTA-3'
V	ITS2	F: 5'-CAATTGAAAACCGCTTAGCGTGTTT-3' R: 5'-TGATCTGAGGTCGACATTTCC-3'

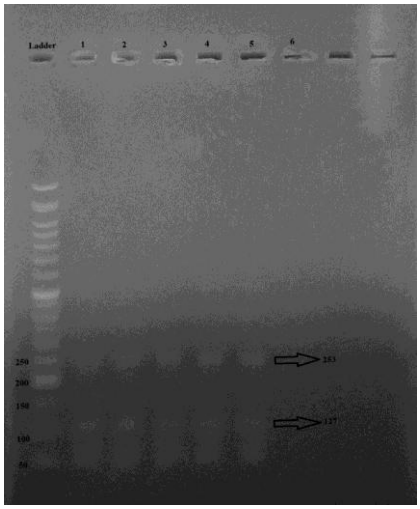

Fig. 2. Following multiplex polymerase chain reaction (PCR) amplification, a two-band pattern was observed *Trichinella britovi* larvae displayed the expected band pattern of 127 bp and 253 bp. The PCR-amplified fragments were visualized by agarose gel electrophoresis (2.00% standard agarose), and the size of the PCR products was estimated by comparing them to a DNA ladder to determine the number of *Trichinella* spp. present. (Gel photo description; Jackal samples from 1: Khorasan Razavi, 2 and 3: Mazandaran, 4: Ardabil, and 5: Positive control, and 6: Negative control.

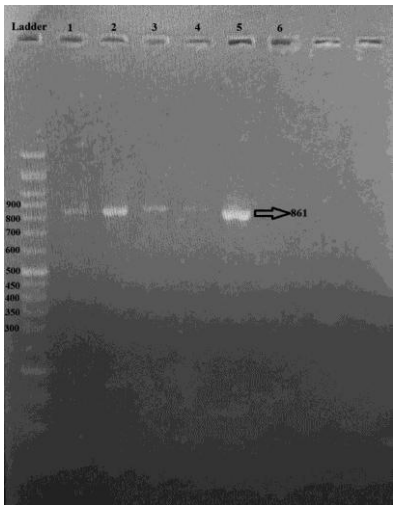

Fig. 3. The polymerase chain reaction products amplified using primers specific to the *cytochrome oxidase subunit I* gene, displaying 861 bp bands in all four samples analyzed. The sequence analysis results were analyzed using the MEGA Software (version 7.0; Biodesign Institute, Tempe, USA) package. (Gel photo description; Jackal samples from 1: Khorasan Razavi, 2 and 3: Mazandaran, 4: Ardabil, 5: Positive control, and 6: Negative control.

Based on the sequencing results from the PCR products, 99.74% similarity was found among the *COX1* gene sequences registered in GenBank® under the accession No. KM100575.1 (Türkiye), MG672269.1 (China), and MN-820641.1 (Iraq). Multiple alignment analysis shows that nucleotide variations within the COX1 sequence of isolates from north of Iran have been observed in a nucleotide sequence analysis of the COX1 sequence. It was discovered that there was a transition (T nucleotide instead of C nucleotide) at position 95 in the nucleotide sequence, but this did not affect the translation of amino acids (Fig. 3).

Phylogenetic analysis. The amplicons obtained from the PCR in this study were approximately 861 bp. The sequences of COX1 obtained from all three individual muscle larvae had 100% identical haplotypes except one nucleotide transversion in one sequence. The nucleotide sequence data obtained in the study were deposited in GenBank® under the accession numbers PP217416 and PP217417. In total, the sequenced PCR products showed identity to *T. britovi* sequences available in GenBank®, including 99.86% similarity with *T. britovi* from Golestan (OK445696, OK445688), 99.72% similarity with *T. britovi* from Razavi Khorasan (OK445698, OK445697), 99.72% similarity with *T. britovi* from India (ON351580), and 99.58% similarity with *T. britovi* from North Khorasan (OK445687, OK445686). A phylogenetic tree based on the similarity of our sequences with those registered in GenBank® showed five groups. The sequences obtained from this study were grouped with *T. britovi* (Fig. 4).

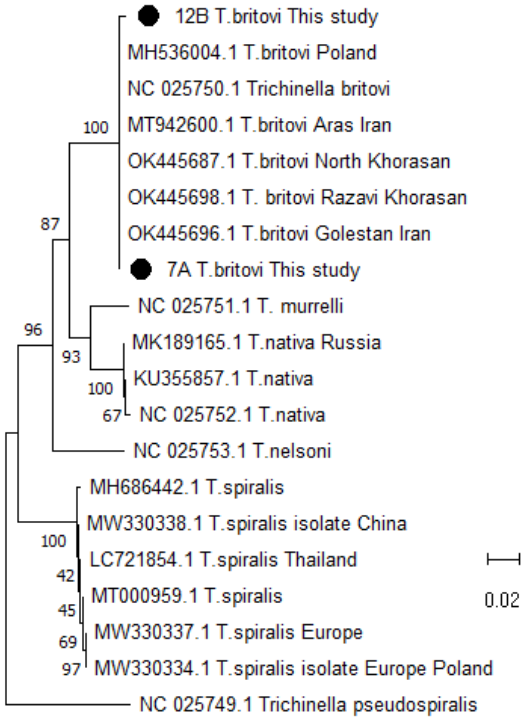

Fig. 4. The amplicons obtained from the polymerase chain reaction in this study were approximately 861 bp. The obtained sequences of cytochrome oxidase subunit I from all four individual muscle larvae had identical haplotypes with the exception of one nucleotide transversion in one sequence. Five primer pairs were used in the multiplex polymerase chain reaction. The nucleotide sequence data obtained in the study have been deposited in GenBank® under the accession numbers PP217416 and PP217417.

Discussion

In Iran, the risk of trichinellosis among wild animals has decreased due to social, economic, and religious factors. However, t sporadic cases still occur among carnivores such as foxes, boars, jackals, and hyenas in some areas. In such circumstances, the infection may emerge and re-emerge in areas that were previously unaffected. This pioneering study fills a critical gap in understanding the prevalence and characteristics of *Trichinella* species within the populations of golden jackals across various regions of Iran. By exploring these previously uncharted territories, the research sheds light on the distribution and potential risk factors associated with Trichinella infection in wildlife populations. This information is essential for developing public health strategies to prevent the spread of Trichinella parasites and protect both animal and human health in these regions.

According to the results of this study, evidence of *T. britovi* was found in four collected samples (4.30%). Multiplex PCR allowed for the estimation of *T. britovi* prevalence in golden jackals in the ecosystem. *T. britovi* is the most frequent species associated with an animal infection, typically found in the northeastern regions of Iran in a subset of stray dogs, with a detection rate of 2.50%.[14] This discovery indicates the potential distribution of *T. britovi* within this geographical area. Previous research in Iran's northern and western territories has also identified *T. britovi* in diverse wildlife species, including a leopard (*Panthera pardus saxicolor*) and a wild boar.[21-24] *Trichinella* spp. infections have been documented in jackals, red foxes, stray dogs, brown bears, wild cats, and wild boars in the Caspian region.[25,26] In the Isfahan region, *Trichinella* spp. infection was detected in stray dogs, jackals, red foxes, stripped hyenas, and one rodent (*Meriones persicus*).[27] Both *T. spiralis* and *T. britovi* have been documented.[28] These findings highlight the wide-ranging presence of *T. britovi* across varied ecological habitats and hint at the possibility of its coexistence with other *Trichinella* species in the region. *T. britovi* is the etiological agent of infection of sylvatic carnivores residing in temperate areas of the Palearctic region from the Iberian Peninsula to Kazakhstan, Iran, and Türkiye.[29] The distribution area of this species likely extends to other Asian countries where information is currently lacking such as India and China. Epidemiological and molecular data also indicate also its presence in the wildlife of the Mediterranean countries in Africa.

Further exploration of the geographical distribution of *Trichinella* species in northeastern Iran is necessary to clarify the full range of parasite diversity and assess associated risks to wildlife and human populations. The need for further investigation using molecular techniques arises from the potential complexities in the current study's findings. While initial results suggest the presence of certain species, molecular techniques offer a more precise and comprehensive approach to species identification. Thus, conducting additional molecular analyses will help confirm the accuracy of the findings presented in this study and provide a deeper understanding of the genetic diversity and distribution of the target species. Since pork and wild boar meat are considered forbidden in Iran due to religious beliefs, few studies have been performed to examine the epidemiology of Trichinella. Several animals, such as jackals, red foxes, stray dogs, brown bears, wild cats, wild boars, striped hyenas, and one rodent (*M. persicus*), have been identified with the disease in Iran's north and central regions.[29]

In addition, there has been a report of human infection based on clinical symptoms, a history of eating undercooked wild boar meat, and low titer circulating antibodies in the patient's serum.[29] A case of trichinellosis has recently been confirmed in Tehran using molecular methods in a family with a history of consuming wild boar meat infected with *T. britovi* in the past.[22,30] Additionally, human infections caused by *T. britovi* have been documented in France, Italy, Spain, and Türkiye as a result of consuming free-range pig meat, game meat, or horse meat that had been raised without a cage.[31,32] The clinical course of *T. britovi* is benign as it produces fewer newborn larvae than *T. spiralis* and there have been no reports mortality. Molecular phylogenetic analysis indicates a close relationship between *T. britovi* in this region and other regions of Iran and Poland. The results of this study provide valuable insight into the prevalence and phylogenetic distribution of *T. britovi* among Iranian golden jackals. Further surveillance, control measures, and public awareness campaigns are needed to combat the problem. Managing and preventing the spread of these parasitic diseases is essential to prevent them from affecting humans and animals in the future.

A wide range of wildlife species can be found in this part of the world. Moreover, additional studies should be conducted in neighboring countries to enhance our understanding of the strains present in those regions. This would provide us with a more comprehensive understanding of the strains found in those countries.

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Conflict of interests

The authors declare no conflict of interest.

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