

Molecular study on parasitic nematodes infection in the abomasum of sheep in Ilam, Iran

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Abstract

Parasitic nematodes of ovine abomasum are of economic and hygienic importance throughout the world and Iran. This study was aimed to evaluate molecular identity and species diversity of parasitic nematodes in the abomasum of slaughtered sheep in Ilam, Iran. In this study, a total number of 240 of abomasa were randomly collected from the slaughtered sheep at industrial slaughterhouses in Ilam in all seasons between 2017 and 2018. The abomasum content and abomasal mucosa were removed and washed. The collected nematodes were morphologically identified. The genomic DNA was extracted and a 300 bp-fragment-length from internal transcribed spacer 2 ribosomal ribonucleic acid (ITS2-rRNA) gene was amplified. Overall prevalence was 66.70% (160/240). Five species of four genera of nematodes including *Marshallagia marshalli* (43.70%), *Ostertagia circumcincta* (15.50%), *Parabronema skrjabini* (5.00%), *M. occidentalis* (2.50%), and *Haemonchus contortus* (0.04%) were identified. *Ostertagia circumcincta* and *H. contortus* were found to be different in two nucleotides. There was no nucleotide difference between *M. marshalli* and *M. occidentalis*. This study revealed a significant prevalence of parasitic nematodes in sheep abomasum and species diversity of Trichostrongylid nematodes in the region.

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Introduction

Strongylida and Spirurida nematodes are common parasites in the abomasum of domestic and wild small ruminants throughout the world and Iran.¹ *Ostertagia circumcincta*, *Haemonchus contortus*, and *Trichostrongylus* spp. have a wide geographic distribution in arid areas while *Marshallagia marshalli* is only found in sheep and goats living in infrequent precipitation condition and steppe climates.^{2,3} In Iran, six species of nematodes, i.e. *H. contortus*, *O. circumcincta*, *M. marshalli*, *O. occidentalis*, *O. trifurcata*, and *Parabronema skrjabini* have been reported from abomasum of sheep in three distinct climate zones of Mazandaran, Isfahan and Khuzestan.⁴ Nematodes infection in the abomasum of herbivorous animals like cattle, sheep and goats are also prevalent in most regions of the world.⁵

Morphologic and morphometric studies of parasites are common methods to identify parasitic nematodes. However, these methods are time consuming and not reliable for identification of adult or other evolutionary

phases, i.e. larvae and eggs up to species and/or strains level. Molecular tools are recently applied for classification and study the genetic diversity of parasitic helminths.^{6,7} The PCR and PCR-RFLP methods have been employed for amplifying. Because of the prevalence of helminth infections and regarding their effect on sheep production, this study was carried out to determine the molecular identity and species diversity of ovine parasitic nematodes in Ilam, Iran.

Materials and Methods

Area of study and collection of abomasum. Ilam is located in Ilam province in the west of Iran, (N 33°38' and E 46°26') surrounded by mountains and its climate is also affected by deserts from the west and the south. Ilam province has a climatic trilogy of cold, tropical and temperate with annual relative humidity 64.00%, relative rainfall of 446.81 mm The relative temperature varies from -12.60 to +40.60 °C in different parts of the

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region. According to Iranian Veterinary Organization (IVO, 2018), an average population of 1,243,000 sheep and lambs are reared in this region. During the study, a total number of 240 abomasa (20 per month) were randomly collected from sheep slaughtered at Ilam industrial slaughterhouse within one year (2017-2018). The abomasa were tied off at both ends and removed to be inspected for nematodes parasites. The nematodes were collected and relaxed at 4.00 °C in physiologic serum (0.85%) for 24 hr. The recovered nematodes were counted and fixed in 70.00% ethanol and cleared by lactophenol. They were identified at magnification 400× and 1000× using identification keys.⁸⁻¹³ The morphologic examination in details was not performed on identified helminths due to focus of the investigation on reliable molecular examinations.

DNA Extraction. For molecular analysis, the soft tissues of the nematodes were dissected, washed several times in 0.01 M phosphate-buffered saline (PBS, pH 7.2), and stored at - 20.00 °C until DNA extraction. Genomic DNA was isolated using SinaPure™ kit (Sina Clone, Iran) following manufacturer's instruction.

Polymerase chain reaction (PCR). A pair of primers, (NC1: 5'-ACGTCTGGTTCAGGGTTGTT-3' and NC2: 5'-TTAGTTTCTTTTCTCGCT-3') were used to amplify a 300bp-fragment-length of the internal transcribed spacer 2 ribosomal ribonucleic acid (ITS2-rRNA) gene.¹⁴ The PCR reaction was carried out in 50.00 µL reaction mixture containing 3.00 µL (100ng) of genomic DNA (diluted 1:30), 25.00 µL 2× Master Mix, and 10.00 pM of each primer, and distilled water (20.00 µL). The reaction was performed in an Eppendorf thermal cycler. The samples were subjected to an initial denaturation step at 95.00 °C for 5 min, followed by 30 cycles of 45 sec at 94.00 °C for, 45 sec at 55.00 °C and 50 sec at 72.00 °C, and a final extension step at 72.00 °C for 5 min.¹⁴ A PCR mixture excluding the DNA was used as a negative control. A volume of 10.00 µL of each PCR product was analyzed by electrophoresis on 1.50% (w/v) agarose gel for approximately 90 min at 90 V. The gels were stained with ethidium bromide 0.50 µg⁻¹ and visualized under UV light.

Phylogenetic analysis. The amplified gene fragments of the selected individuals belonging to *M. marshalli*, *H. contortus*, *M. occidentalis*, and *O. circumcincta* were sequenced by Bioneer Corporation (Daejeon, South Korea) using an automated sequencer (3730xl/Bioneer 3730xl).

Both forward and reverse sequences were assembled and edited using SeqMan II module of Lasergene (version 7.1; DNA-STAR, Madison, USA). Also, sequence divergence of the isolates was calculated using MegAlign module of the same package. The resulting sequences together with 12 reference sequences data of nematodes isolated from abomasa of sheep worldwide were aligned using Clustal W. phylogenetic tree. They were constructed using maximum likelihood method and applying Kimura 3-parameter model¹⁵ in MEGA X Software (version 10.0; Biodesign Institute, Tempe, USA).¹⁶ This analysis involved 15 nucleotide sequences. All positions containing gaps and missing data were eliminated (complete deletion option). There were a total number of 260 positions in the final dataset. The tree was rooted using *Trichostrongylus axei* (accession no AY439026) as the out-group. The statistical significance of branching orders was calculated by the bootstrap resampling process (1000 replicates).

Statistical evaluation. The data were analyzed using one-way ANOVA and Post Hoc LSD tests with a confidence interval of 95.00% (SPSS version 22.0, IBM Corp, Armonk, USA). Probability of ≤ 0.05 was regarded as significant. Data was presented as mean number of isolated parasite per sheep ± standard deviation (mean ± SD).

Results

Frequency and species diversity of nematodes.

Data of the frequency at different seasons are shown in Table 1. Overall, 160 out of 240 examined abomasa (66.70%) were positive for nematodes infection with 22,728 nematodes (9,262 males and 13,467 female). The highest infection rate of nematodes was found in the spring (76.70%, 46/60). The identified species of nematodes, the frequency and mixed infection rates are given in Table 1.

Laboratory identification indicated that there were five species of four genera of nematodes including *M. marshalli* (43.70%, 120.20 ± 28.50), *O. circumcincta* (15.50%, 62.00 ± 16.70), *M. occidentalis* (2.50%, 5.17 ± 1.40), *P. skrjabini* (5.00%, 10.15 ± 6.40), and *H. contortus* (0.04%, 0.22 ± 0.03). Mixed infections with 2(28.30%), 3(12.10%) and 4(2.90%) nematodes species were identified (Table1). The highest number of *M. marshalli* ($p < 0.05$, 120.20 ± 28.50) and *O. circumcincta* ($p < 0.05$, 62.00 ± 16.70) was found in fall (Table 1).

Table 1. Seasonal distribution of nematodes isolated from abomasum of sheep sampled in Ilam city, West of Iran (mean ± SD, n = 240).

Time (season)	No. infected animals (%)	No. nematodes Isolated (%)	<i>Haemonchus contortus</i>	<i>Parabronema skrjabini</i>	<i>Ostertagia circumcincta</i>	<i>Marshallagia occidentalis</i>	<i>Marshallagia marshalli</i>
Spring	46 (76.70)	3853 (17.00)	0.20 ± 0.10	3.50 ± 2.20	10.20 ± 2.40 ^a	3.20 ± 1.50	47.00 ± 13.80 ^{ac}
Summer	32 (53.30)	1914 (8.30)	0.03 ± 0.01	8.20 ± 4.40	7.60 ± 2.10 ^a	3.40 ± 1.70	12.80 ± 3.40 ^a
Autumn	41 (68.30)	11814 (52.00)	0	10.20 ± 6.40	62.00 ± 16.70 ^b	5.20 ± 1.40	120.20 ± 28.50 ^b
Winter	41 (68.30)	5147 (22.50)	0	2.90 ± 1.50	12.30 ± 4.60 ^a	2.7 ± 1.20	67.90 ± 19.50 ^c

^{abc} Different letters in each column shows the statistical significance ($p < 0.05$).

Molecular and phylogenetic analysis. All samples were successfully amplified and yielded fragments of about 300 bp (Fig. 1). Comparisons of the present sequences with other available records in GenBank®, revealed that some isolates had great similarity (more than 99.00%) with *H. contortus*, and the others had high homology with *M. marshalli*, *M. occidentalis*, and *O. circumcincta*. Some of sequences were deposited in GenBank® database under the accession numbers MK760915-MK760919.

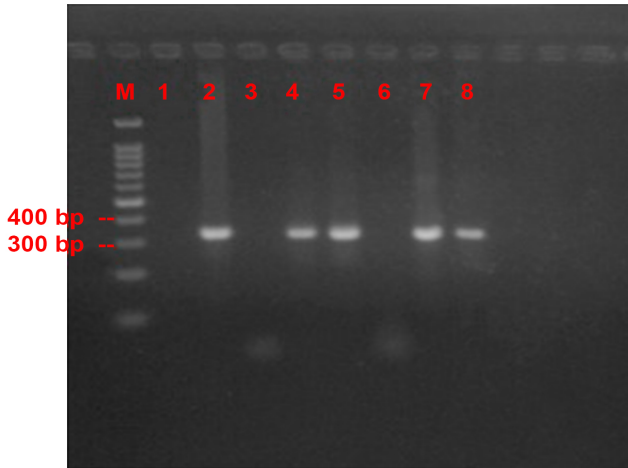


Fig. 1. Agarose gel electrophoresis of ITS2 –rRNA gene of the abomasum nematodes. Lanes 4: *M. marshalli*; 5: *M. occidentalis*; 7: *O. circumcincta*; 8: *H. contortus*; Lanes 1, 3, 6: negative control; Lane M: 100bp DNA size marker.

Compared to most closely related records, the intra-species divergence for isolates of *H. contortus*, *M. marshalli*, *M. occidentalis*, *O. circumcincta* were 0.00-1.80%, 0.00%, 0.00%, and 1.10-5.50%, respectively. While inter-species sequence variation among sheep nematodes was significantly higher, ranged from 0 to 26.90% (Fig. 2). According to the phylogenetic tree (Fig. 3), all species of the present study were grouped in a cluster with the relevant reference sequences from previous studies.

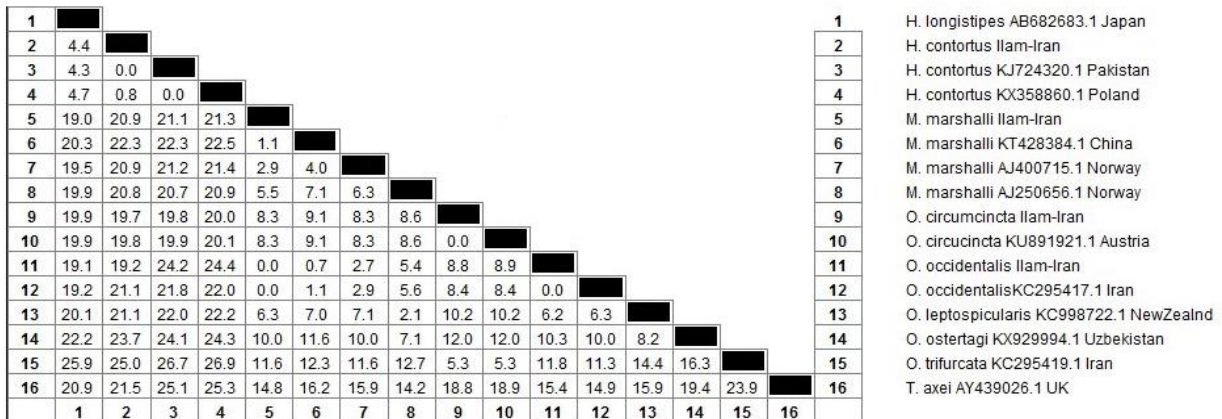


Fig. 2. ITS2-rRNA sequence divergence of representative selection of nematodes in sheep abomasum.

Discussion

Information on the prevalence of helminths is essential to implement effective control measures. The prevalence rates of nematodes infections in the abomasum of examined sheep in Ilam were comparable to those reported from different parts of Iran, i.e. Tabriz (88.00%),¹⁷ Fereidoonkenar (62.00%),¹⁸ Mazandaran, Isfahan, Khuzestan (30.89%),⁴ and Shahrekord (26.70%).¹⁹ High prevalence of the parasites in sheep was also reported from Turkey (100%),²⁰ Ethiopia (94.50%)²¹ and Pakistan (49.47%).^{22,23}

In the present study, the occurrence of nematodes infections had a seasonal pattern which started in April with a peak in spring and gradually stepped down during summer. In contrast, the highest infection rate of nematodes reported in infected sheep of Khoy, northwest of Iran, was occurred in summer.²⁴ In neighboring countries of Iran, e.g. Baluchistan, Pakistan, the highest prevalence of nematodes infections in abomasum of examined sheep was reported in fall (65.68%).^{22,23} In Turkey, nematodes infection was increased during the early and late spring.²⁰ This might be as a result of varying weather conditions, i.e. relative rainfall, temperature and humidity, the eggs or larvae density in a pasture, excessive grazing in wet and marshy areas, anthelmintic drugs prescription and genetic resistance.^{25,26}

The present investigation elucidated that *M. marshalli* was prevalent strongyloid nematodes in sheep. In Iran, the predominant reported species of nematodes in sheep abomasum were *M. marshalli* (6.70%) in center,¹⁹ *O. circumcincta* (39.00%) in southwest,²⁶ *M. marshalli* (38.00%) and *O. circumcincta* (38.00%) in the north¹⁸, *Haemonchus* spp. (77.20%), *M. marshalli* (46.00%), *O. occidentalis* (39.00%) and *P. skrjabini* (22.00%) in the west.²⁷⁻²⁹ The prevalent species of the nematodes in neighboring and other countries was *H. contortus* from Iraq (71.36%),¹⁰ Pakistan (71.36%),²² *O. circumcincta* (80.00%) from Turkey²⁰ and *H. contortus* (90.00%) from Ethiopia.²¹ These differences might be due to geographical and environmental conditions, habits of definitive hosts, husbandry and feeding principles and livestock management system.^{18,21-23}

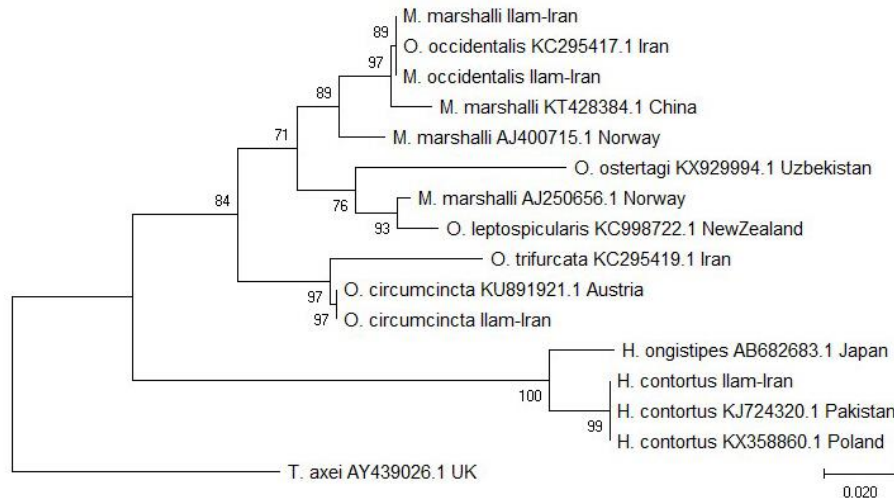


Fig. 3. Phylogenetic tree of nematode isolates of sheep abomasum obtained in this study and reference sequences retrieved from GenBank® based on ITS2-rRNA nucleotide sequences and constructed using the Tamura's 3-parameter model.

Phylogenetic analysis using DNA sequence data is considered as a powerful approach for the construction of the evolutionary relationships among different groups of organisms including the parasitic helminths.³⁰ However, the utility of this method seems to be dependent on the target DNA fragment and the rates of sequence divergence among the organisms remains unknown.³¹ The constructed phylogenetic tree using the sequences of ITS2-rRNA from diverse localities fractionate of identified nematodes had polyphyletic positions on the tree. So far, in Iran only a few molecular studies of ITS2-rRNA in nematodes were carried out to characterize the parasitic nematodes in the abomasum of ruminants.^{1,8,9} In the current study, *M. marshalli* and *M. occidentalis* showed identical sequences. This was in agreement with Dallas *et al.* who noted this finding for examined abomasa of reindeer (*Rangifer tarandus platyrhynchus*).³² The molecular analyses revealed the presence of two distinct *O. circumcincta* and *H. contortus*. *Ostertagia* spp. that played an intrinsic diversity role with a comprehensive hosting capacity and a significant geographic distribution. Sabor Darbandi *et al.* reported that *O. circumcincta*, *O. occidentalis*, and *O. trifurcata* had 2.00 - 3.00% and 97.00 - 98.00% differences in nucleotide sequences and homology, respectively.⁹ In a series of phylogenetic studies, Meshgi *et al.* revealed the genotypic and phenotypic diversity in *H. contortus* and *H. longistipis* with two distinct species in Iran.¹ Roeber *et al.* reported exact consistency between microscopic detection and molecular study of *O. circumcincta* and *H. contortus*.³³

Marshallagia marshalli and *O. circumcincta* were the prevalent nematodes in sheep abomasa in this region in spring. In this regard, effective parasites control and further studies for a better understanding of the geo-graphic and seasonal distributions of the parasites are necessary. The traditional morphologic and morphometric indices were

insufficient for species identification of nematodes isolates. Regarding the polyphyletic positions of the sequences from the isolates, discriminative morpho-logic criteria and their application together with a combination of molecular tools like phylogenetic analysis may be a helpful approach for the construction of the evolutionary relationships among different groups of nematodes and alterations in their epidemiological patterns.

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Conflict of interest

The authors declare there is no conflict of interests.

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