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Original paper

Molecular study on nematode infection in sheep abomasa: a regional investigation in Iran and Iraq

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ABSTRACT. Gastrointestinal nematodes are leading causes of loss in livestock and are the primary restriction to its profitable production, worldwide. This study was carried out to determine the prevalence and diversity of sheep abomasum nematode species in Urmia (Iran) and Soran (Iraq) slaughterhouses from October 2019 to January 2021. A total of 280 abomasa (each city 140 samples) were randomly collected from the slaughtered sheep. The abomasal content and mucosa were removed and washed. The collected nematodes were morphologically identified. Genomic DNA was extracted from identified nematodes and a fragment from the internal transcribed spacer 2 ribosomal ribonucleic acid (ITS2-rDNA) gene was amplified. In Urmia city, two species including *Teladorsagia circumcincta* (40.7%), and two morphotypes of *Marshallagia* species; *Marshallagia marshalli* (35.0%) and *M. trifida* (4.3%) were identified. In Urmia city, 52.9% of the examined sheep were infected with at least one species of nematodes. The overall prevalence of abomasa infection with nematodes in Soran city was 91.4%. In the examined sheep abomasa in Soran city, four species of nematodes were identified, including *Marshallagia* species with two morphotypes, *M. marshalli* (85.0%) and *M. trifida* (20.7%), *Teladorsagia circumcincta* (32.1%), *Parabronema skrjabini* (1.4%), and *Haemonchus contortus* (0.7%). Except for *H. contortus*, all the other identified nematode species were confirmed using molecular techniques. It was concluded that abomasal nematode infections are widespread in sheep particularly in Soran city. *Marshallagia marshalli* and *T. circumcincta* were most prevalent nematodes in both regions. In addition, further molecular studies are recommended to understand the intra-specific variations in the genus *Marshallagia* and more accurate identification of morphotypes in these regions.

Keywords: nematode, sheep, ITS2-rDNA, Iran, Iraq

Introduction

In developing countries of the Middle East, parasitic infections have considerable impact on the quality of lives in human and livestock. By stealing nutritional resources via several pathways, parasites have a remarkable impact on one billion people worldwide, who rely on livestock for their livelihoods [1]. Among parasitic infections in livestock, nematodes are probably the main health challenge in ruminants. By threatening the health and welfare of domestic animals, nematode infections undermine livestock production. They are omnipresent and principally all grazing livestock are exposed to nematode infections [2]. Trichostrongylid nematodes are parasites of the digestive tract of ruminants, with many species having dramatic effect on health of human as well, especially on those who live in close contact with infected animals [3,4]. *Haemonchus contortus* feed on blood of host animal, and heavy infection with it causes severe anemia and even death in the animal. Other nematodes as *Trichostrongylus* spp. and *Teladorsagia circumcincta* inflict damage to gastrointestinal cells and to digestion mechanism [3,4].

Several morphological and morphometric methods are employed to identify parasitic

nematodes. Faecal egg counts through microscopy is a common method to monitor gastrointestinal helminth infections. However, this method is time-consuming and often falls short of correct identification of adult nematode species, especially that of the trichostrongylids, due to the apparently similar eggs and larvae [5]. Molecular techniques such as PCR, PCR-RFLP and phylogenetic analysis are applied for the classification of parasitic helminths and studying their genetic diversity [5].

Internal transcribed spacer (ITS) 1 and 2 regions in ribosomal DNA (rDNA) are widely studied regions in phylogenetic analysis of gastrointestinal nematodes [6]. Understanding the diversity and epidemiology of the digestive tract nematodes on pasture are necessary for the launch of control programs [7]. Livestock husbandry is an important economic dependency of people living in Urmia (north-west Iran) and Soran (north-east Iraq). Yet, there is a lack of information about species diversity of nematodes in the abomasa of sheep in these regions. The current study was aimed at identifying species diversity of helminth parasites in the region by applying both morphological and molecular tools.

Materials and Methods

Study areas and nematode collection/identification

In the present study sheep abomasa were collected from slaughtered sheep at slaughterhouses in Urmia (Iran) and Soran (Iraq). These two regions have an estimated three million and two million sheep, respectively [8,9]. During the course of this study, a total of 280 abomasa (each city 140) were randomly collected from slaughtered sheep (158 males and 122 females). The abomasa were tied off at both ends and transferred to the Parasitology laboratory at Urmia University, Iran and Biology laboratory at Soran University, Iraq. The recovered nematodes from examined sheep abomasa were relaxed at 4°C in physiologic serum (0.85%) for 24 h. Worms were counted, then fixed in 70% ethanol, and finally cleared in lactophenol for identification [10,11].

DNA extraction and PCR amplification of ITS2-rDNA

Soft tissues of identified nematodes were fixed in 70% ethanol and then they were dissected and washed several times in phosphate-buffered saline (0.01M, pH 7.2). The washed soft tissues were stored at −20°C until they were used for DNA extraction. Genomic DNA extraction was performed for each identified species using DNA Extraction kit DNP (SinaClon, Iran) according to the manufacturer's instruction. Specific primers including forward (5′-CCTGGTTAGTTTCTTTTC CTCCGC-3′) and reverse primers (5′-CGGTGGA TCA CTCGG CTCGT-3′) for *P. skrjabini* and NC1 (5′-ACGTCTGGTTCAGGGTTGTT-3′) and NC2 (5′-TTAGTTTCTTTTCCTCCGCT-3′) for *T. circumcincta*, *M. marshalli* and *M. occidentalis* were used for the amplification of the ITS2-rDNA gene [12,13]. PCR reaction was prepared in 50 µl reaction mixture containing 5 µl (100ng) of genomic DNA, 25 µl 2× Master Mix (Pishgaman-Iran), and 2 µl of each primer (concentration: 10 pm/µl) and distilled water (16 µl). The PCR reaction was performed in peqStar 96 Universal gradient thermal cycler (Peqlab, Germany). The samples were subjected to an initial denaturation step at 95°C for 5 min, followed by 35 cycles of 30 sec at 95°C, 30 sec at 50°C and 1 min at 72°C, and a final extension step at 72°C for 7 min [14]. The thermal cycles for the amplification of ITS2-rDNA gene from *P. skrjabini* was programmed as an initial denaturation step at 94°C for 3min, followed by 40 cycles of 95°C for 30 sec, 50°C for 30 sec, and 72°C for 1 min, and a final extension step at 72°C for 7 min [12]. A volume of 10 µl of each PCR product was analyzed by electrophoresis on 1.5% (w/v) agarose gel. The gels were stained with ethidium bromide (0.5 µg ml-1) and visualized under UV light (FastGene® FAS-DIGI PRO-Germany).

Nucleotide sequencing and phylogenetic analysis

The amplified PCR fragments from each nematode species except for *H. contortus* were sent to Kawsar Biotech Company (Iran) for nucleotide sequencing. The PCR fragments were sequenced from both directions. Both forward and reverse sequences were assembled and edited using SeqMan II module of Lasergene (version 7.1; DNA-STAR, Madison, USA). The sequence divergence of the isolates was calculated using MegAlign module in the same package. The obtained sequences from the present study were aligned and compared with the deposited ITS2 rDNA sequences of nematodes in GenBank using Clustal W. Phylogenetic tree was constructed using maximum likelihood method by applying Kimura 3-parameter model in MEGA X Software (version 10.0; Bio design Institute, Tempe, USA) [15,16]. All positions containing gaps and missing data were eliminated (complete deletion option). The statistical significance of

Table 1. Prevalence and species diversity of nematodes in abomasa of slaughtered sheep in Urmia and Soran cities

	Nematodes	No. of infected sheep		
		Male*	Female*	Total*
Urmia (Male=105) (Female=35) (Total=140)	*M. marshalli*	56 (40%)	18 (12.9%)	17 (12.1%)
	T. circumcincta			19 (13.6%)
	M. marshalli and *T. circumcincta*			32 (22.9%)
	M. trifida and *T. circumcincta*			6 (4.3%)
Total		74 (52.9%)		74 (52.9%)
Soran (Male=53) (Female=87) (Total=140)	*M. marshalli*	45 (32.1%)	83 (60.1%)	57 (40.7%)
	T. circumcincta			7 (5%)
	M. trifida			2 (1.4%)
	M. marshalli and *T. circumcincta*			33 (23.6%)
	M. marshalli and *M. trifida*			21(15%)
	M. marshalli and *P. skrjabini*			2 (1.4%)
	M. marshalli, *M. trifida* and *H. contortus*			1 (0.6%)
	M. marshalli, *M. trifida* and *T. circumcincta*			5 (3.6%)
Total		128 (91.4%)		128 (91.4%)

* Percentage of infected animals calculated from total overall (n=140)

branching orders was calculated by the bootstrap resampling process (1000 replicates).

Statistical analysis

Statistical analyses were performed using SPSS statistics version 22.0 (IBM Corp., NY, USA). The differences between groups were evaluated by Chi-square test. A *P*-value of less than 0.05 was considered statistically significant.

Results

Frequency of nematode infection

Almost two-third (72.2%) of examined sheep abomasa were infected with nematodes. Four different species of abomasum nematodes were identified (Fig. 1). *Marshallagia marshalli* was the most prevalent specie, infecting 168 (60.0%) of the examined sheep, followed by *T. circumcincta* infecting 102 (36.4%) sheep (Tab. 1). The frequency of infection in female sheep (82.3%) was significantly higher than male sheep (63.9%) ($P<0.05$).

Out of 140 collected abomasa from Urmia city, infected male and female sheep were 40.0% and 12.9%, respectively. Two species of nematodes including *T. circumcincta* and two morphotypes of *Marshallagia marshalli* (*M. marshalli* and *M. trifida*) were identified. *Teladorsagia circumcincta* (40.7%) was the most prevalent nematode infecting sheep, followed by *M. marshalli* (35.0%) (Tab. 1).

The prevalence of nematode infection in Soran was 91.4% which was significantly higher than nematode infection of sheep abomasa in Urmia city (52.9%) ($P<0.05$). In Soran city *M. marshalli* was the predominant nematode infecting the abomasa (Tab. 1). The frequency of infected male and female sheep with abomasum nematodes were 45 (32.1%) and 83 (60.1%) respectively, with significant difference ($P<0.05$) (Tab. 1). Four species of nematodes were identified, i.e., *M. marshalli* (both morphotypes), *T. circumcincta*, *Haemonchus contortus* and *Parabronema skrjabini*. *Marshallagia marshalli* was the predominant specie in abomasa of sheep in Soran city infecting 85% of all examined sheep followed by *T. circumcincta* (32.1%). *Haemonchus contortus* and *P. skrjabini* were found in one and two sheep respectively in this area. Co-

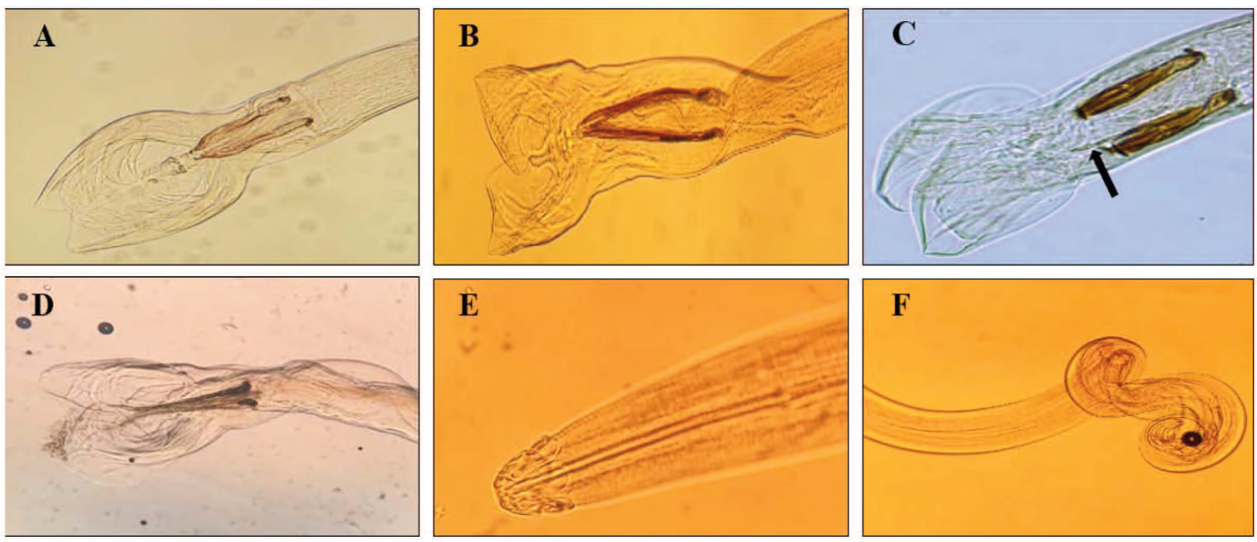

Figure 1. Adult forms of detected nematodes in slaughtered sheep in Urmia and Soran cities: (A) Copulatory bursa and spicules of *M. marshalli*; (B) Copulatory bursa and spicules of adult male of *T. circumcincta*; (C) Copulatory bursa, spicules and less conspicuous gubernaculum (arrow) of *M. trifida*; (D) Copulatory bursa and fused spicules in adult male of *H. contortus*; (E) Existence of cuticular shields and cordons in the cephalic region of *P. skrjabini*; (F) Spiral tail in adult males of *P. skrjabini*

infection of nematodes was observed in 62 (48.4%) abomasa, with co-infection of *M. marshalli* and *T. circumcincta* being observed in 33 (25.8%) abomasa.

Molecular and phylogenetic findings

DNA extracted from all morphologically identified nematodes except for *H. contortus* produced fragments of 309 bp for *P. skrjabini* and 272 bp for other species. Comparisons of the ITS2-rDNA sequences in the present study with other available ITS2 sequences in GenBank, indicated that identified nematodes had great similarity (more than 99.00%) with nematodes in the other parts of the world. Sequences from the nematodes identified in the present study were deposited in GenBank with the following accession numbers: *M. marshalli* isolate MMUrmia: MZ148588, *M. trifida* isolate MOUrmia: MZ148613, *T. circumcincta* isolate TCUrmia: MZ148623, *M. marshalli* isolate MMSoran: MZ148587, *M. trifida* isolate MOSoran: MZ148614, *P. skrjabini* isolate PSSoran: MZ151415, and *T. circumcincta* isolate TCSoran: MZ148615. Seven nematode sequences of the present study were grouped in two clusters with the relevant reference sequences from previous studies (Fig. 2).

Discussion

Knowledge of the prevalence and diversity of helminths in small ruminants is essential to launch control programs. The prevalence of nematode infection in the abomasa of examined sheep in Urmia and Soran cities were comparable to the other reports from Iran, Iraq, Turkey, Saudi Arabia, and Syria [17–23]. In Garmiyan and Kurdistan provinces of Iraq, *M. marshalli* was the most prevalent parasite in small ruminants [20]. In Erbil province, Iraq, *H. contortus* (22%) and *M. marshalli* (4.18%) were reported as prevalent species in the examined sheep [24]. In Iran and Syria *M. marshalli* was the predominant infecting nematode with the prevalence of 65.4% and 81%, respectively [17,23]. However, in examined animals, *T. circumcincta* (40.71%) was found to be prevalent nematode in Urmia, Iran. In other countries, it was also *T. circumcincta* in Turkey (80%) [22], while it was *H. contortus* in Pakistan (71.36%) [25]. These differences in the prevalence of sheep abomasum nematodes may be due to climate condition. It has been noted that the relation between rainfalls and nematode infection was negative for *Marshallagia* as the highest prevalence of *Marshallagia* infection was in dry areas at the end of the dry-season or in the beginning of rainy season [26].

Obtained results also indicated that the rate of infection in females was higher than male sheep ($P<0.05$). This is in agreement with the previous studies [27–29] and could be due to the stress during pregnancy, peri-parturient period and lactating

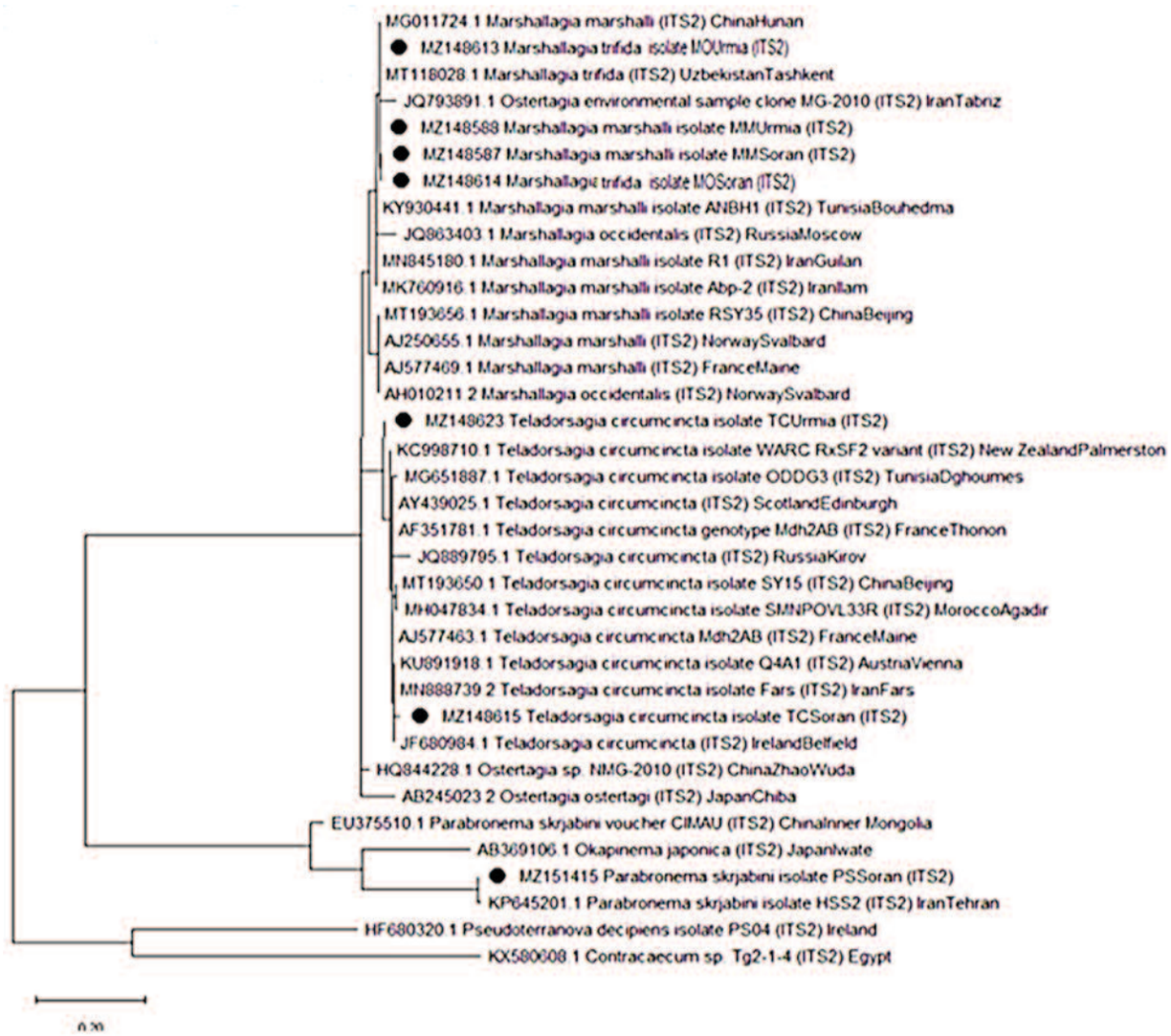

Figure 2. The evolutionary history was inferred using the Maximum-likelihood method. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The analysis involved 36 nucleotide sequences

leading to suppression of the immune system. Genetic predisposition and differential susceptibility owing to hormonal control in male and female animals can influence the susceptibility of livestock to nematode infections [30,31].

Molecular biology assays, such as PCR and DNA sequencing provide reliable identification of gastrointestinal nematodes up to the species level in small ruminants [32]. Several ribosomal and mitochondrial regions in the genome have been used for the recognition of nematodes. However, the ITS2 gene region has been used as a valuable genetic marker for identification of gastrointestinal nematodes, especially at generic level [33]. The comparison of the ITS2 sequences of the nematode isolates in the current study with the other existing ITS2 sequences of the abomasa nematodes in the GenBank showed that *M. marshalli* isolate in Urmia had 100% homology with *M. trifida* in Uzbekistan Tashkent (MT118028.1), 92.7% homology with *Ostertagia* from Tabriz, Iran (JQ793891.1) and 86.5% with *M. marshalli* reported in Hunan, China (MG011724.1). *Teladorsagia circumcincta* isolate in Urmia city had high homology with *T. circumcincta* in New Zealand (95.2%; KC998710.1) and *T. circumcincta* (96.9%, AY439025.1) reported in Scotland. Moreover, *M. trifida* in Urmia had a high level of similarity with *M. trifida* in Uzbekistan (91.7%, MT118028.1), *M. marshalli* in China (89.6%, MT193656.1), and *O. ostertagi* in Japan (91.1%, AB245023.2).

ITS2 gene sequence of *M. marshalli* isolate in Soran city showed 90.1% homology with ITS2 gene of *M. marshalli* from Norway (AJ250655.1) and

95.7% similarity with *Ostertagia* spp. Isolated from China (HQ844228.1). ITS2 gene similarity of *T. circumcincta* from Soran city was 97.9% with *T. circumcincta* (MN888739.2) from Fars Province, Iran, and 98.8% with *T. circumcincta* (JF680984.1) from Belfield, Ireland. The partial ITS2 gene of *P. skrjabini* isolate from Soran city showed 71% homology with ITS2 gene of *P. skrjabini* (EU375510.1) from China, Inner Mongolia. ITS2 sequence of *M. trifida* from Soran city uncovered 91.4% homology with ITS2 gene of *M. marshalli* isolate from China (MT193656.1) and 92.9% similarity with *Marshallagia trifida* (MT118028.1) isolate from Uzbekistan.

Few ITS2 gene sequences are available in Genbank for *P. skrjabini* to compare with the current study. *Parabronema skrjabini* from Soran city was clustered with *P. skrjabini* from China, Inner Mongolia (EU375510). ITS2 region of the *Parabronema* is highly variable even in specimens that are isolated from the same host [34].

Sequencing of higher number of nematodes from different parts of the world and also sequencing of other regions of the nematode genome such as 28S rRNA and cox1 genes are necessary for a better understanding of inter- and intra-specific similarities. Although the objective of the ITS2 analysis in present study was to confirm the species diversity, however, our phylogenetic analysis showed that the morphotypes of *Marshallagia* were grouped together in one clade with high level of homology (e.g. *M. marshalli* isolate had 100% homology with *M. trifida*) highlighting that the ITS2 region is a suitable marker for exploring the intra-specific polymorphism within the genus *Marshallagia*. This is not in agreement with the previous studies in Iran and Uzbekistan reporting that ITS2 fragment is inappropriate marker to distinguish morphotypes in some trichostrongyloid nematodes particularly *Marshallagia* species [17,35–37].

In conclusion, according to the findings in the present study, the prevalence of nematodes infections was high particularly in female sheep and the species diversity of nematodes in abomasa of examined sheep was remarkable. *Teladorsagia circumcincta* and *M. marshalli* are respectively the most abundant species of nematodes in the abomasum of the examined sheep in Urmia and Soran cities. In addition, the findings of this study revealed that the burden of infection and the diversity of species in Soran city were higher than Urmia city. The current study was the first epidemiologic investigation and molecular identification of sheep abomasal nematodes in Soran region, Iraq which can be essential towards a better knowledge of gastrointestinal parasitic infection among small ruminants in the region.

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