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Original Article

Comparison of Internal Transcribed Spacers and Intergenic Spacer Regions of Five Common Iranian Sheep Bursate Nematodes

Reza NABAVI [1], Brendan CONNEELY [2,3], Elaine MCCARTHY [2], Barbara GOOD [3], Parviz SHAYAN [4], * Theo DE WAAL [2]

1. *Dept. of Pathobiology, Faculty of Veterinary Medicine, University of Zabol, Zabol, Iran*
2. *School of Veterinary Medicine, University College Dublin, Belfield, Dublin 4, Ireland*
3. *Teagasc, Animal Production Research Centre, Athenry, Co Galway, Ireland*
4. *Dept. of Parasitology, Faculty of Veterinary Medicine , University of Tehran, Tehran, Iran*

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***Correspondence**
Email:
theo.dewaal@ucd.ie

Abstract

Background: Accurate identification of sheep nematodes is a critical point in epidemiological studies and monitoring of drug resistance in flocks. However, due to a close morphological similarity between the eggs and larval stages of many of these nematodes, such identification is not a trivial task. There are a number of studies showing that molecular targets in ribosomal DNA (Internal transcribed spacer 1, 2 and Intergenic spacer) are suitable for accurate identification of sheep bursate nematodes. The objective of present study was to compare the ITS1, ITS2 and IGS regions of Iranian common bursate nematodes in order to choose best target for specific identification methods.

Methods: The first and second internal transcribed spacers (ITS1and ITS2) and intergenic spacer (IGS) of the ribosomal DNA (rDNA) of 5 common Iranian bursate nematodes of sheep were sequenced. The sequences of some non–Iranian isolates were used for comparison in order to evaluate the variation in sequence homology between geographically different nematode populations.

Results: Comparison of the ITS1 and ITS2 sequences of Iranian nematodes showed greatest similarity among *Teladorsagia circumcincta* and *Marshallagia marshalli* of 94% and 88%, respectively. While *Trichostrongylus colubriformis* and *M. marshalli* showed the highest homology (99%) in the IGS sequences. Comparison of the spacer sequences of Iranian with non-Iranian isolates showed significantly higher variation in *Haemonchus contortus* compared to the other species.

Conclusion: Both the ITS1 and ITS2 sequences are convenient targets to have species-specific identification of Iranian bursate nematodes. On the other hand the IGS region may be a less suitable molecular target.

Introduction

In Iran, as in many countries in the world, bursate nematodes belonging to the Trichostrongyloidea superfamily are of major veterinary importance in small ruminant production systems (1, 2). *Ostertagia* species and *Marshallagia marshalli* are the most prevalent nematodes infecting sheep and goats (3). Iran is a semi dry country but climatologically can be divided into 4 different zones. Zone 4 (Central and Salt Deserts) is neither suitable for animal husbandry nor fit for human habitations. The other three zones include: Caspian zone (Zone I), Mountain plateau zone (Zone II) and the Persian Gulf Lowland (Zone III). The combined sheep and goat populations in these 3 zones are 52 million and 26 million, respectively (2, 4). Anthelmintic drug resistance has become a serious and widespread problem throughout the world. Recent reports indicate that anthelminthic resistance also occurs in some sheep nematodes, especially in *Teladorsagia circumcincta,* in Iran (5, 6).

Accurate identification of sheep nematodes is a critical point in epidemiological studies and monitoring of drug resistance in flocks (7-9). However, due to a close morphological similarity between the eggs and larval stages of many of these nematodes, such identification is not a trivial task (7, 10). There are a number of studies showing that ribosomal DNA (rDNA), especially the internal transcribed spacer (ITS) 1 and 2 regions, are suitable regions for phylogenetic investigations and valuable targets to design probes or define markers for the identification of bursate nematodes (8, 10-13). Some studies have also successfully used the intergenic spacer (IGS) fragment for species-specific identification of strongyle nematodes (14, 15). Due to genetic diversity and also intraspecific variations among nematodes from different geographical populations (9, 16), it seems advantageous to have complete sequences of the genetic targets to be used in species identification from all target species from a specific geographical region.

In this study, five common bursate nematodes (*Haemonchus contortus, Trichostrogylus colubriformis, Teladorsagia circumcincta, Marshallagia marshalli, and Nematodirus oiratianus*) were collected from sheep of zones II and III of Iran. The objective was to compare the ITS1, ITS2 and IGS regions of these nematodes in order to choose best target for specific identification methods.

Materials and Methods

Nematode samples

H. contortus, T. colubriformis, T. circumcincta, M. marshalli, N. oiratianus adult male nematodes were collected from the gastrointestinal tract of sheep slaughtered from Khuzestan, Chaharmahal and Bakhtiari provinces in southwest Iran. Nematodes were washed in phosphate buffered saline (PBS) and adult male nematodes were identified morphologically according to the keys of Soulsby (17) and then stored in 70% ethanol until being used. Before DNA extraction, the nematodes were removed from ethanol, dried and washed in distilled water and stored for 1–2 days in 1.5 ml tube at −20 °C.

DNA extraction and PCR

High Pure PCR template preparation kit (Roche Diagnostics) was used to extract DNA from a single male nematode of each of the species following the manufacturer's instructions. The DNA of the samples was stored at -20 °C until used.

Available GenBank 28s and 18s sequences of *H. contortus* (AM039742), *T. colubriformis* (AJ920350 and AM039743.1), *Nematodirus battus* (AJ920360 and AM039752) and *T. circumcincta* (AF044934.1) were used to design primers. To amplify the ITS1, 5.8S and ITS2 regions in one reaction, the primers were

based on conserved sequences at the 3'end of 18s rDNA and 5'end of 28s rDNA (Table 1). The IGS primers were designed from conserved sequences at the 3'end of the 28S rDNA and 5'end of 18s rDNA (Table 1).

The PCR was performed on 50 µl total volume and included 1x PCR buffer (Promega), 1U Taq polymerase (Promega), 30 pmol/50 µl of each primer (SIGMA), 200 µM of each dNTP(Promega), 3.5 mM $MgCl_2$ and approximately 2 ng per 4 µl of genomic DNA in an automated thermocycler (Thermohybaid, MSC), (18) under the following conditions: 5 min incubation at 95 °C to denature double-strand DNA, 35 cycles of 45 s at 60 °C (annealing step), 45 s at 72 °C (extension step), and 45 s at 94 °C (denaturing step). Finally, PCR was completed with an additional post-amplification extension step for 10 min at 72 °C. Samples without genomic DNA were included with each amplification as negative controls. The PCR products were analyzed on a 1% agarose gel in 1× TAE buffer and visualized using SYBR-Green dye and UVP Image Analyzer (BioSpectrum®AC Imaging System).

Table 1: The primers used in amplification of ITS1, 5.8s, ITS2 and IGS regions. F: Forward primers, R: Reverse primers

Species		Forward and reverse primers used to amplification of ITS1, 5.8s, ITS2 (5'-3')		Forward and reverse primers used to amplification of IGS (5'-3')
Haemonchus contortus	F	GCGGGAAACAGTTCAATCGC	F	ACCGTCGTGAGACAGGTTAG
	R	TCCCCGTTCACTCGCCGTTA	R	CTTAGACATGCATGGCTTAATC
Teladorsagia circumcincta	F	GCGGGAAACAGTTCAATCGC	F	ACCGTCGTGAGACAGGTTAG
	R	TCCCCGTTCACTCGCCGTTA	R	CTGCTCTAATGAGCCGTTCG
Marshallagia marshalli	F	GCGGGAAACAGTTCAATCGC	F	GCGACGTTGCTTTTTGATCC
	R	TCCCCGTTCACTCGCCGTTA	R	CTGCTCTAATGAGCCGTTCG
Trichostrongylus colubriformis	F	GCGGGAAACAGTTCAATCGC	F	ACCGTCGTGAGACAGGTTAG
	R	TCCCCGTTCACTCGCCGTTA	R	CTGCTCTAATGAGCCGTTCG
Nematodirus oiratianus	F	GTAGGTGAACTGCGGAAGGATCATT	F	ACCGTCGTGAGACAGGTTAG
	R	TTAGTTTTCCTCCGCT	R	CTGCTCTAATGAGCCGTTCG

PCR product purification and cloning

To purify the PCR products, 40 µl of each PCR reaction mix was loaded on a 1 % low melting point agarose gel. The specific amplified fragments bands (800-1000bp in ITS and 600-1500 bp in 28s, IGS and ETS) were cut out and purified with Promega DNA purification system kit. PGEM-T Easy Vector cloning kit (Promega) was then used following the manufacturer's instructions to clone the purified PCR products. The vectors are prepared by cutting the pGEM®-T Easy Vectors, with EcoRI and adding a 3´ terminal thymidine to both ends. Insertional inactivation of the alpha-peptide allows recombinant clones to be directly identified by blue/white screening on indicator plates. PureYield™ Plasmid Miniprep System (Promega) was used for plasmid purification before being sent for sequencing (GATC-biotech, Germany).

Sequencing and data analysis

Two samples were sequenced in both directions and consensus sequences derived for each species. Multiple alignments of the ITS1, ITS2 and IGS sequences for each species were then used to compare and calculate similarity scores between species. In this step rDNA sequence of some non-Iranian nematode isolates were also included (Table 2). No additional IGS sequences were available in Genbank for comparison. ClustalW2 sequence alignment tool (EMBL-EBI - http://www.ebi.ac.uk/Tools/msa/clustalw2/) was used for all alignments and calculation of similarity score.

Table 2: Accession numbers of the ITS1, ITS2 and IGS sequences used as comparison in the present study.

Nematode Species (Origin)	ITS1, Accession no	ITS2, Accession no	IGS, Accession no
Haemonchus contortus (Iran)	HQ389229	HQ389229	HQ389234
Teladorsagia circumcincta (Iran)	HQ389230	HQ389230	HQ389235
Marshallagia marshalli (Iran)	HQ389231	HQ389231	HQ389236
Trichostrongylus colubriformis (Iran)	HQ389232	HQ389232	HQ389237
Nematodirus oiratianus (Iran)	HQ389233	HQ389233	HQ389238
Haemonchus contortus (Africa)	JF680983	JF680983	JF680983
Teladorsagia circumcincta (UK)	JF680984	JF680984	JF680984
Trichostrongylus colubriformis (UK)	JF680985	-	-
Haemonchus contortus (Genbank)	EU0846911	EU0846911	-
Teladorsagia circumcincta (Genbank)	AF044934.1	AY439025.1	-
Marshallagia marshalli (Genbank)	AY013242.1	AJ577469.1	-
Trichostrongylus colubriformis (Genbank)	Y15876.1	AB503242.1	-

Results

ITS1 and ITS2 were identified from the whole ITS1-5.8S-ITS2 sequenced fragment for each species. The length of ITS1 and ITS2 fragments ranged between 382-400 and 231-248 bp, respectively (Fig. 1; Table 3).

The IGS was identified from the whole 28S-IGS-ETS-18S sequenced fragment for each species and ranged between 179 (partial sequence) – 457 (complete sequence) bp (Fig. 2; Table 3). Sequence data of the ITS1, ITS2 and IGS of *H. contortus* (Africa), *T. circumcincta* (UK) and ITS1 of *T. colubriformis* (UK), also sequenced as part of another study in University College Dublin, was also included in the final comparison (Table 2, 4 & 5). Similarity score percentage in the ITS1, ITS2 and IGS regions are summarized in Table 4 & 5. Because of incomplete sequences in the ITS2 and IGS of *T. colubriformis* (UK) it was not included in the analysis. Overall, the highest similarity in the ITS1 sequences was detected among *T. circumcincta* and *M. marshalli* (94%) and the lowest between *N. oiratianus* and *H. contortus* (Africa) (67%).The Iranian and UK *T. circumcincta* and *T. colubriformis* ITS1 sequences were 100% identical. The similarity score percentage between the Iranian nematodes and equivalent species obtained from GenBank ranged between 96% - 99% (Table 4).

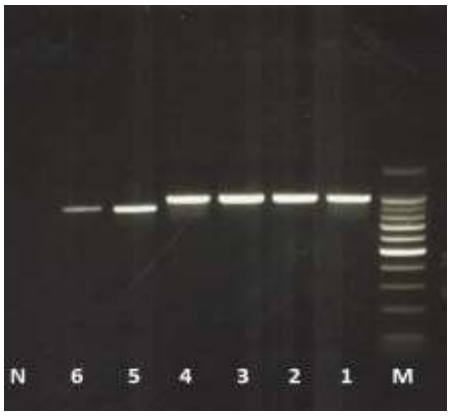

Fig. 1: Amplified total fragment of ITS1-5.8S-ITS2 for **1:** *Haemonchus contortus,* **2** and **3:** *Teladorsagia circumcincta,* **4:** *Marshallagia marshalli,* **5:** *Nematodirus oiratianus*, **6:** *Trichostrongylus colubriformis,* **N** is negative control and **M** is 100 bp marker

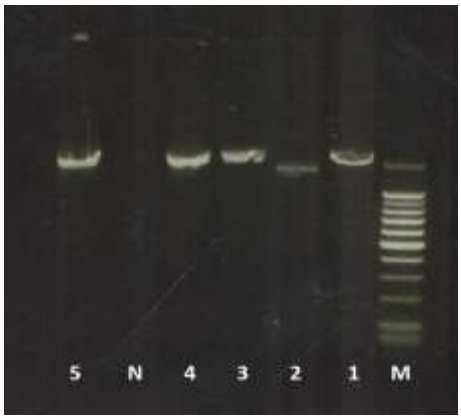

Fig. 2: Amplified total fragment of IGS-ETS for **1:** *Teladorsagia circumcincta,* **2:** *Haemonchus contortus,* **3:** *Nematodirus oiratianus*, **4:** *Trichostrongylus colubriformis,* **5:** *Marshallagia marshalli,* **N** is negative control and **M** is 100 bp marker

In the case of the ITS2 sequences the similarity score percentage among the Iranian nematodes and the equivalent species obtained from GenBank ranged between 96% - 100% (Table 4). Iranian *H. contortus* and *H. contortus* (EU0846911) were 100% identical. The highest similarity among the genera overall was detected between *T. circumcincta* and *M. marshalli* (88%) and the lowest similarity was between *N. oiratianus* and *H. contortus* (Africa) (64%).

The IGS sequence comparison revealed some unexpected results. A high similarity was found between *T. colubriformis* and *M. marshalli* (99%) and low similarity between *T. circumcincta* (UK) and all other species. The highest similarity was between *T. circumcincta* and *M. marshalli* (78%) and lowest similarity was between *N. oiratianus* and *T. circumcincta* (47%) (Table 5).

Table 3: The length of sequenced ITS1, ITS2 and IGS regions in Iranian isolates

Species	**ITS1**(bp)	**ITS2**(bp)	**IGS**(bp)
Haemonchus contortus	400	231	380
Teladorsagia circumcincta	391	248	179*
Marshallagia marshalli	383	237	457
Trichostrongylus colubriformis	387	240	457
Nematodirus oiratianus	382	238	207*

*Partial sequence

Table 4: Similarity score percentage of ITS1 and ITS2 sequences of studied nematodes and some available sequences from GenBank.

ITS2 / **ITS1**	*Haemonchus contortus* (Iran)	*Teladorsagia circumcincta* (Iran)	*Marshallagia marshalli* (Iran)	*Trichostrongylus colubriformis* (Iran)	*Nematodirus oiratianus* (Iran)	*Haemonchus contortus* (Africa)	*Teladorsagia circumcincta* (UK)	*Trichostrongylus colubriformis* (UK)	*Haemonchus contortus* (EU0846911)	*Teladorsagia circumcincta* (AY439025.1)	*Marshallagia marshalli* (AJ577469.1)	*Trichostrongylus colubriformis* (AB503242.1)
Haemonchus contortus (Iran)	-	78	78	77	65	97	78	-	100	82	79	77
Teladorsagia circumcincta (Iran)	84	-	88	83	65	75	99	-	78	98	86	82
Marshallagia marshalli (Iran)	83	94	-	83	65	75	88	-	78	90	96	83
Trichostrongylus colubriformis (Iran)	83	86	86	-	69	76	83	-	77	84	82	99
Nematodirus oiratianus (Iran)	69	73	72	71	-	64	66	-	65	69	67	69
Haemonchus contortus (Africa)	97	84	83	83	67	-	75	-	97	80	77	76
Teladorsagia circumcincta (UK)	84	100	94	86	73	84	-	-	78	99	86	82
Trichostrongylus colubriformis (UK)	83	86	86	100	71	83	86	-	-	-	-	-
Haemonchus contortus (EU0846911)	96	85	85	85	68	97	85	85	-	82	79	77
Teladorsagia circumcincta (AF044934.1)	84	99	92	87	75	84	99	87	85	-	91	84
Marshallagia marshalli (AY013242.1)	82	92	96	85	71	81	92	85	83	90	-	82
Trichostrongylus colubriformis (Y15876.1)	83	87	87	98	71	83	87	98	85	88	85	-

Table 5: Similarity score percentage of IGS of studied nematodes

IGS	*Haemonchus contortus* (Iran)	*Teladorsagia circumcincta* (Iran)	*Marshallagia marshalli* (Iran)	*Trichostrongylus colubriformis* (Iran)	*Nematodirus oiratianus* (Iran)	*Haemonchus contortus (Africa))*	*Teladorsagia circumcincta (UK)*
Haemonchus contortus (Iran)	-	-	-	-	-	-	-
Teladorsagia circumcincta (Iran)	68	-	-	-	-	-	-
Marshallagia marshalli (Iran)	57	78	-	-	-	-	-
Trichostrongylus colubriformis (Iran)	57	76	99	-	-	-	-
Nematodirus oiratianus (Iran)	66	47	68	72	-	-	-
Haemonchus contortus (Africa)	93	69	59	58	60	-	-
Teladorsagia circumcincta (UK)	15	28	17	15	43	34	-

Discussion

Previous studies have indicated that sequences of the ITS and IGS regions of rDNA are useful targets to find genetic markers (7-9, 15) for the differentiation between nematode species. In the present study we have shown that there are differences in the ITS regions between the five species that could be utilized for specific identification (Table 4). On the other hand the IGS region may be a less suitable molecular target for some taxa, compared with the ITS regions, as there can be considerable length variation in the IGS sequence within individual organisms (9). The extent of sequence similarities of the IGS region among the Iranian species in this study ranged from 47 to 99 %. A very high sequence homology (99 %) was observed between *T. colubriformis* and *M. marshalli,* whereas the similarity between *T. circumcincta* (UK) and all other species was low (15-28 %). Low levels of similarity between related species and extensive sequence homology between species of different genera was also observed in horse cyathostomins (19).

Several studies have been carried out on rDNA of bursate nematodes. Most studies have found that the sequence variation in both the ITS1 and ITS2 sequences within species is quite small (9, 12). However, there are limited data available on the genetic variation among different nematode population from different geographical regions. In the case of *H. contortus,* 15 nucleotide variations were found in the ITS1 region between isolates from Iran and Africa. This included 10 nucleotide substitutions and 5 nucleotide insertions/deletions. Furthermore the length of ITS1 showed 3bp differences. The ITS1 of Iranian *T. circumcincta* and *T. colubriformis* were completely identical to the respective UK isolates and is in general agreement with results from Hoste et al. (10) who also found no differences between *T. colubriformis* isolates. Similarly, the ITS2 sequences of the two *H. contortus* isolates showed 8 nucleotide variations, including 6 nucleotide substitutions and 2 nucleotide inser-

tions/deletions. In the case of *T. circumcincta* only 2 nucleotide substitutions were observed (Table 4).

Conclusion

Both the ITS1 and ITS2 sequences are convenient targets for the species-specific identification of Iranian bursate nematodes. Due to some sequence variations that may occur between geographically different isolates (of the same species), it is advisable to obtain sequences from local isolates before design of specific identification methods.

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