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Original Article

Phylogenetic Study of *Haemonchus* Species from Iran Based On Morpho-Molecular Characterization

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Abstract

Background: Haemonchosis has a negative effect on the farming industry throughout the world, especially in the tropic and sub-tropic countries. The present study was carried out to differentiate *Haemonchus* species from its main hosts in Iran, including sheep, goat and camel.

Methods: The identification took place based on the morphometrics of the spicules and molecular characters. Two hundred seventy adult male nematodes were collected from the abomasums of different ruminants (90 samples from each animal) at the slaughterhouses from different localities in Iran. Samples were morphologically identified according to the spicules' morphometric measurements. In the section on molecular study, 10 samples of each *Haemonchus* isolates were genetically examined. A simple PCR-restriction fragment length polymorphism (PCR-RFLP) assay of the second internal transcribed spacer of ribosomal DNA (ITS2-rDNA) were described to confirm the PCR results.

Results: PCR-RFLP profile obtained from the restriction enzyme *HPa1* in *H. contortus* and *H. longistipes* indicated 1 (278 bp) and 2 (113 and 135 bp) different fragments, respectively. The morphological parameters clearly distinguish *H. contortus* from *H. longistipes*. Moreover, regarding the ITS2-rDNA, sequences of 295 bp and 314 bp were obtained from *H. contortus* and *H. longistipes*, respectively.

Conclusion: The genotypic results are in agreement with the phenotypic findings of both species.

Introduction

Haemonchus spp. (Nematoda: Trichostrongyloidea), commonly known as the "Wire Worm" or "Barber Pole Worm" causes cosmopolitan helminthiasis and shows wide distribution among ruminants. This abomasa nematode is responsible for the economic losses due to anemia, weight loss and reduced milk production. The prevalence rate of haemonchosis in Iran has highly declined in the past two decades (1). Considering the rise in temperature and light rain during the past decades along with mass treatment and control strategies are responsible for this event. As the result of the drug resistance, epidemiological parameters, control strategies and pathological future, it is important to differentiation between *Haemonchus* species. Despite the importance of morpho-molecular structures in *Haemonchus* spp. it is the limited amount of information in Iran.

Samson-Himmelstjerna et al. recommended analysis of ITS2 sequences in several trichostrongylide nematodes (2). Heise et al. characterized the ITS2 of the eight gastrointestinal nematodes of ruminants (3).

The genetic status of *Marshallgia marshalli* and *M. occidentalis* based on the ITS2 sequencing was studied and both species are conspecific (4). Moreover, in a similar study a complete ITS2 and part of 28S-rDNA were used to distinguish *M. marshalli* from *Ostertagia gruehneri* (5). Sequence data of the ITS is extensively used and it's a high-resolution marker for several parasitic nematodes (3-6). Important morphological characters of *Haemonchus* species were described in previous investigations. For example, cuticular ridge patterns (7), length of the spicules (8) and novel morphometric parameters for identifying individual speciments of *Haemonchus* spp. in adult male and female in ruminants of North America (9).Considering the specimens in mixed infections, hybrids of different species, morphological similarity of the species, and the absence of morphomolecular data on this abomasal nematodes in Iran, more studies are required to confirm the identity of some *Haemonchus* species.

According to the above information, this study aimed to distinguish *H. contortus* from *H. longistipes*based on ITS2-ribosomal DNA in sheep, goat and camel isolates as well as to identify the morphometrics of spicules in the prevalent species of *Haemonchus* in the different ruminants in Iran.

Materials and methods

Collection parasite

Two hundred seventy adult male *Haemonchus* were collected from the abomasa of naturally infected animals which included sheep (HST, HSG and HSK, n=90), goat (HGT and HGK, n=90) and camel (HCT and HCI, n=90). The sampling was performed at local abattoirs from four different provinces in Iran; Tehran, Isfahan, Khuzestan and Gilan (Table 1).

Table 1: *Haemonchus* isolates used in the present study

Parasite code	Host	Geographical origin	N. of worms	Identity
HST	Sheep	Tehran	30	*H. contortus*
HSG	Sheep	Gilan	30	*H. contortus*
HSK	Sheep	Khuzestan	30	*H. contortus*
HGT	Goat	Tehran	40	*H. contortus*
HGK	Goat	Khuzestan	50	*H. contortus*
HCT	Camel	Tehran	60	*H. longistipes*
HCI	Camel	Isfahan	30	*H. longistipes*

The worms repeatedly were washed with a phosphate buffer saline solution and were labeled using the abbreviated names of the *Haemonchus*, host and collection site. They were, then, stored in 70% ethanol at room temperature until use.

Morphological identification

A total of 180 *Haemonchus* spp. from sheep, goat and camel isolates were cleared in phenol-alchohol and examined for spicule length and right/left spicule barber length in male worms (8, 9). In order to check for statistic analyse, *t*-student test and SPSS 18 software were adopted. A *P*-value of <0.05 was considered to be significant.

DNA extraction and PCR amplification of ITS2

Thirty samples of *H. contortus* from sheep, goat and camel were characterized molecularly. These samples were collected from different geographic localities in Iran. Genomic DNA was extracted from individual worms using a DNA isolation kit (MBST, Iran), following the manufacturer's recommendation. The worms were rigorously crushed using a sterile scalpel for about 5 min. the crushed worms were, then, laysed in 180 µl of laysis buffer in a 1.5 ml tube. Protenase K (20 µl) was added and the mixture was incubated for 10 min. at 55°C. DNA samples from each isolate were liquated and stored at -70°C. Genomic DNA was amplified by PCR using specific primers HcI-F: 5`CTC-GTC-TGG-TTC-AGG-GTT3` (forward) and HcI-R: 5`GTA-ACC-TCG-CTG-AGC-TCA3` (reverse) for ITS2 region. The primers were designed according to the available nucleotide sequence data from ITS2 in GenBank (accession no. HQ844231.1).

PCR volume was 100 µl containing 1×PCR buffer, 2 mM MgCl2, 2 mM dNTPs, 0.4 µmol of each primer, 2.5 unite/µl Taq DNA polymerase (Cinnagen) and 1 µl DNA sample in an automated thermocycler (Biorad-Italia). The PCR was performed under the following conditions: 5 minutes of denaturation at 94°C followed by 35 cycles of 45 s at 94 °C, 58°C (annealing), 45 s at 72 °C (denaturation) and final extention of 72°C for 5 min. Samples without genomic DNA were included in each amplification run as the negative controls. A 1000 bp ladder molecular weight marker (Vivantis-Romania) were included on each gel for base-pair comparison. PCR products were resolved in 1.5% agarose gels, stained for 10 minutes in ethidium bromide, detected on a trans-illuminator and photographed digitally.

PCR-RFLP

Specific restriction enzyme *HPaI* was selected for distinguishing *H. contortus* from *H. longistipes*. According to the instructions provided by the manufacturer, the PCR products were purified from each sample using a DNA kit (MBST, Iran). ITS2 PCR production of both *Haemonchus* species were purified and digested using 1 µl of *HPa1* (10 UI) restriction enzyme (Fermenras) at 37 °C for overnight. The restriction fragments were analyzed using electrophoreses on 2% agarose gels, stained and photographed upon trans-illumination.

Sequence analysis and phylogeny

The purified PCR products were sequenced for all samples (Kawsar Biotech Co. Iran). The obtained sequences were analyzed using the program called Chromas software and, subsequently, aligned with the Clustal W2 software. Sequences available in the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/) were included in the final alignment for comparison with the obtained sequences. It was no conflicting results between the PCR – RLFP and Sequencing methods.

Pairwise comparisons were conducted of the level of sequence differences (D) using the formula D=1-(M/L), where M is the number of alignment positions at which the two sequences have a base in common, and L is the total number of alignment positions over which the two sequences are compared (10).

Results

Morphometric parameters

Although the spicules' size and the distance of terminal spine are only minor differences between *H. contortus* from sheep and goat, these parameters were significant (*P*<0.05) for differentiation of *H. contortus* from *H. longistipes* (Table 2).

Posterior end of the adult male*H. contortus* and *H. longistipes*are shown in Fig. 1.

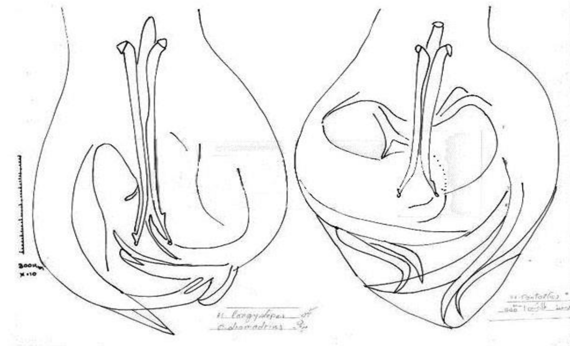

Fig. 1: Copulatory bursa and spicules in *H. contortus* (right) and *H. longistipes*(left)

Table 2: Mean of the morphometric data of *Haemonchus* isolates from three different hosts*

Species	Host	Number examined	Spicule length (µ)	Spicule barb length (µ)	
				Right	Left
H. contortus	Sheep	30	388-550 (473±35)	31-56 (45±7)	20-45 (32±7)
H. contortus	Goat	30	382-490 (448±23)	32-51 (44±4)	19-30 (23±2.5)
H. longistipes	Camel	30	526-689 (645.5±30.6)∫	59-120 (96.4±12)∫	32-48 (37.9±5.2)∫

* Mesurements are Min-Max ranges followed by Mean±SD in parentheses.
∫ Mesurements that differ significantly (95% confidence) from other species.

PCR amplification and RFLP

DNA amplification of the ITS2-rDNA produced a single fragment of 295 bp for all the *H. contortus* from sheep and goat (Fig 2, left), while a 314 bp in length were obtained for the *H. longistipes* samples (Fig. 2, right).

RFLP patterns of *H. contortus* and *H. longistipes* were analyzed after digestion of the PCR products by *HPa1* restriction enzyme. Digestion of the PCR products samples using RFLP method is shown in Fig. 3.

In Fig. 3, two different fragments, 17 and 278 bp (left), and three fragments, 66, 113 and 135 bp (right), were obtained using the restriction enzyme *HPa1* in *H. contortus* and *H. longistipes,* respectively (the fragments of 17 and 66 in both species were not observed).

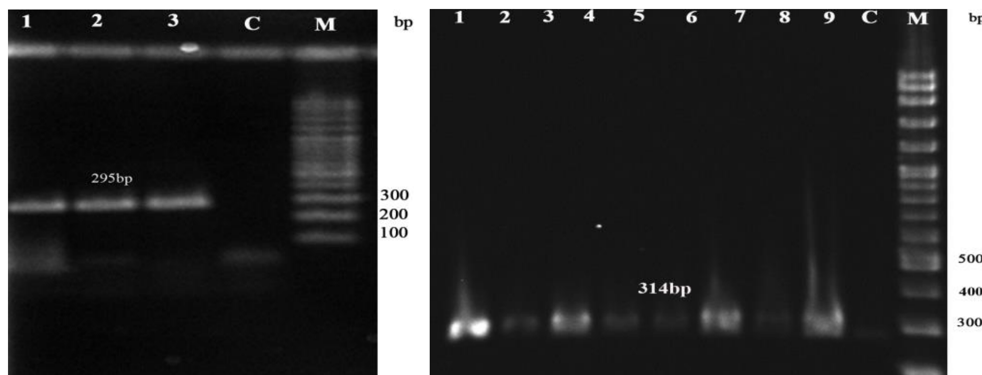

Fig. 2: Agar gel electrophoresis of ITS2 PCR product of *H. contortus* (left) of sheep (Lane 1&2) and goat (Lane 3) and *H. longistipes* (right) of camel (Lane 1-9) from the isolates in Iran, C: negative DNA control, M: DNA marker

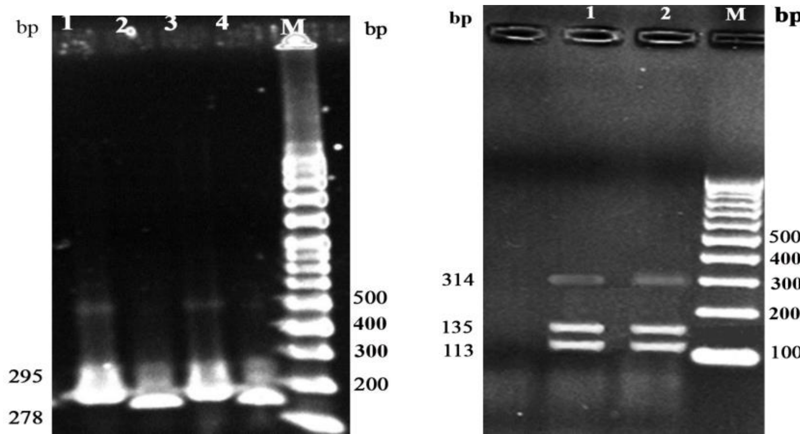

Fig. 3: Restriction enzyme pattern of the ITS2 region of *H. contortus* from the sheep isolates (left, Lane 1&2) and goat (left, Lane 3&4) using *HPaI* represent 1 fragments of 278 bp (Lane 1&3 undigested and Lane 2&4 digested) and of *H. longistipes* from camel isolates (right, Lane 1&2) represent 2 fragment of 113 and 135 bp, M: DNA marker

Sequencing analysis

The second internal transcribed spacer of ribosomal DNA of all the *Haemonchus* isolates was sequenced. A comparison of the sequence identified in this study with the previously recorded *H. contortus* (of sheep or goat) and *H. longistipes* (of camel) nucleotide sequences in the GenBank showed 100% similarity (Accession no. HQ844231.1 and AB682683, respectively).

Despite the fact that differences in 16 nucleotides between *H. contortus* and *H. longistipes* were observed, all the ITS2-rDNA complete sequence of *H. contortus* and *H. longistipes* showed to be 100% identical with the data sequence presented in the GenBank database (Fig. 4).

MP analysis, based on the complete sequences of ITS2 gene marker, showed that *Haemonchus* isolates could be classified in two clades of *H. contortus* and *H. longistipes* (Fig. 5).

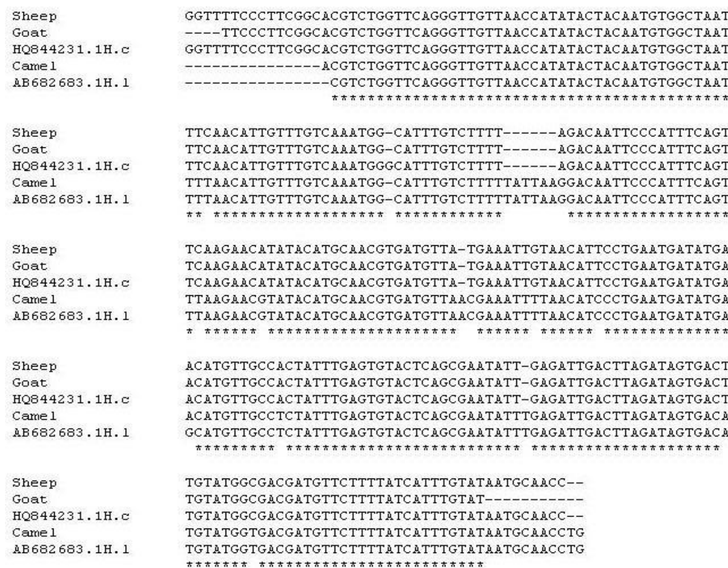

Fig. 4: Alignment of the ITS2 nuclear ribosomal DNA sequences (5`to 3`) of *H. contortus* of sheep and *H. longistipes* of camel, by Clustal W2,Conserved nucleotides are indicated with asterisks and gaps are indicated with hyphens. AB682683.1 isolated from camel and HQ 844231.1 isolated from sheep

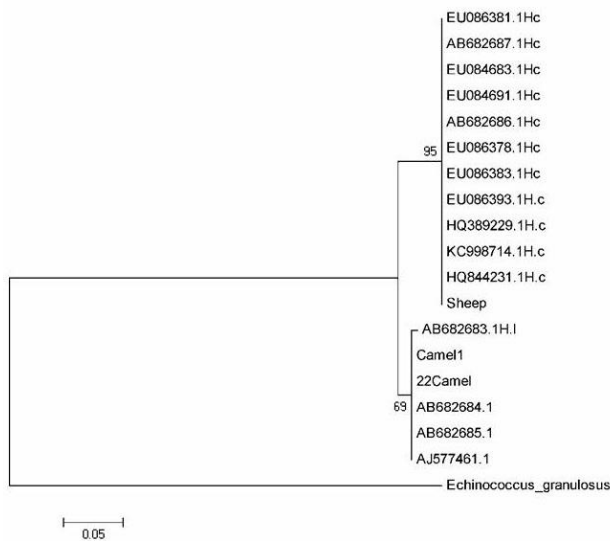

Fig. 5: Phylogenetic relationships of *Haemonchus* parasite based on ITS2 sequences, *Echinococcus granulosus* (AB77790801) was used as an out-group. Phylogenetic analysis were conducted in Mega 5

Discussion

Strongylid nematodes represent the most important parasites of the digestive tract of wild and domestic ruminants in the world. *Haemonchus* spp. is of a major veterinary importance in large and small ruminants. Recent studies have shown a high degree of heterogeneity in this abomasa nematode. Molecular and morphological data on the Iranian *Haemonchus* is very poor. Molecular approaches are needed to study of the phenomena such as host parasite interaction, genetic diversity, drug resistance and molecular polymorphisms.

Many studies in the molecular research of trichostrogylid nematodes have been conducted based on the nuclear rDNA, particularly from the ITS gene (11, 12).

Nabavi et al. and Meshgi et al. studied the prevalence and intensity of abomasal helminths in sheep from three climatic zones (1, 13). Their results revealed that in recent years, significant decrease has occurred in alimentary helminthiasis of the small domestic ruminants in Iran. The prevalence of *H. contortus* in Iran has been reported to be 49% (14) and 3.62% in sheep (1), 6.7% in goat (13) and 0.8% in wild sheep (15).

In this study, the morpho-molecular characteristics of *H. contortus* and *H. longistipes* from three different climatic zones (four provinces) and host isolates in Iran were determined Stevenson et al. reorganized *H. placei* and *H. contortus* using the ITS2 gene marker (16). Their results showed that three (1.3%) nucleotide differences are present between the ITS2 sequences of these species. The comparison of the nucleotide sequences of ITS-2 of *H. contortus* and *H. placei* from Uzbekistan revealed differences in six (2.6%) nucleotides (6). There are not any samples isolated from cattle in the present study. Findings of this study showed that the spicule lengths and the distance of the terminal barb from the spine of the right and left spicules in *H. longistipes* are statistically more significant than *H. contortus* ($P<0.05$). Moreover, there is morphologically significant difference in the spicules size between the *H. contortus* and the *H. longistipes*. In addition, it was found that based on the morphometric and molecular findings there is no difference between *H. contortus* from sheep or goat isolates; and it seems that *H. contortus* adapts to different hosts, such as sheep and goat.

A nucleotide difference of 5.1% (16/314) to 5.4% (16/295) was observed between *H. longistipes* and *H. contortus* from the Iranian isolates at 16 variable sequence positions in the ITS2-rDNA. Sequence of ITS2 ribosomal region indicated that the *Haemonchus* spp. could be categorized into two groups, *H. longistipes* from the came lisolate and *H. contortus* from the sheep or goat isolate from Iran. Moreover, no variation was observed in the composition of the obtained ITS2-rDNA sequences and the sequences deposited in the GenBank.

PCR-restriction fragment length polymorphism with endonuclease *HPaI* has different digestion pattern in two *Haemonchus* species. The ITS2 PCR product of *H. contortus* was digested using *HPa1*. A band of approximately

278 was revealed; whereas, two bands of approximately 135, 113 were produced for the *H. longistipes* samples. Therefore, digestion of PCR products with *HPa1*endonuclease could be used to differentiate between the two *Haemonchus* species. Stevenson et al. detected 2 diagnostic sites for the endonucleases BfaI and FokI for description of the *H. placei* and the *H. contortus* (16). In the present study, the PCR products from all of the *H. contortus* samples of sheep and goat were revealed as a single band of 295 bp in size. Whereas, the one step assay of the *H. longistipes,* with a 314 bp band and using HcI-F and HcI-R primers, could detect a suitable differentiation between the *H. contortus* and the *H. longistipes*. ‘this pair of primers could introduce as a validate and reliable primer with respect to the target gene.

No significant correlation was observed between the spicule characteristics in the *H. contortus* isolated from sheep and goat. The results obtained from these morphometric parameters of the spicule were confirmed using sequence analysis. No discrepancies were found between the results of the RFLP and the morphological criteria.

Conclusion

Indeed, the morphometric data, if accurately measured, could give precise results. Researchers have to be attentive to these methods and get equally good economic results. Although, the present research is the first report on molecular study and morphological characteristics of the *Haemonchus* species in Iran, it has important implications for the population's biology and genomic patterns as well as control of the *Haemonchus* species. However, further studies are needed using greater samples from various geographical origins.

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