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Morpho-Molecular Characterization of Cattle *Haemonchus* Nematodes From Southeast of Iran

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Abstract

BACKGROUND: Haemonchosis is one of the most important nematode infections in cattle population. Knowledge of genetic diversity and morphological analysis can provide a foundation for understanding the drug resistance, epidemiological features, and control strategies in a geographical area.

OBJECTIVES: The aims of this study were to evaluate the morphological parameters and molecular targets (ITS1-ITS2 and Beta-tubulin) of cattle *Haemonchus* nematodes from southeast of Iran and to find species diversity and benzimidazole resistance.

METHODS: From May 2016 to April 2017, 300 abomasa of cattle slaughtered at slaughterhouses of Zabol, Zahedan, and Iranshahr were inspected. Ninety-eight adults male *Haemonchus* nematodes were morphologically analyzed. For molecular analysis, the amplification of ITS1, 5.8S and ITS2 regions, and Beta-tubulin fragment was done.

RESULTS: All specimens were morphologically identified as *H. contortus*. The ITS2 sequencing and the phylogenetic tree revealed 99% similarity between *H.contortus* from this study and those from other parts of the world. There was no mutation in the positions and all nematodes were benzimidazole susceptible.

CONCLUSIONS: Our results showed that although *H. placei* is the most common *Haemonchus* species in the cattle around the world, *H. contortus* is mostly prevalent in southeast region of Iran. Moreover, it seems that the induction of benzimidazole resistance is less important in many parts of the world.

KEYWORDS: Benzimidazole, Cattle, *Haemonchus*, Molecular detection, Morphology

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Introduction

Haemonchus species (Trichostrongyloid nematode) are economically important nematodes living in the abomasa of small and large ruminants. The most common species affecting the cattle around the world are *Haemonchus placei* and *H. contortus*, respectively (Achi *et al*., 2003; Fitzpatrick *et al*., 2013). In Iran, prevalence of haemonchosis was 22% (Eslami and Nabavi, 1976), 9.3% (Tehrani *et al*., 2012), 16.2% (Rahimi *et al*., 2020), and 0.22% (Nazarbeigy *et al*., 2021). However, there is no comprehensive information about the geographical distribution of *Haemonchus* throughout the country. Many investigations have shown the morpho-molecular diversity of *Haemonchus* population (Vadlejch *et al*., 2014; Salle *et al*., 2019). Knowledge of genetic diversity and morphological analysis can provide a foundation for understanding the drug resistance, epidemiological features, and control strategies in a geographical area (Meshgi *et al*., 2015; Salle *et al*., 2019). Based on a morphological study, some parameters including total body length, spicule length, gubernaculum length, spicules, spine conditions, and number of longitudinal ridges can be used for the identification of *Haemonchus* species (Nabavi, 2017). Many researchers have indicated that Internal Transcript Spacers (ITS) especially ITS2 are the most convenient targets of molecular approach in the identification of the parasite species (Li *et al*., 2016; Nabavi *et al*., 2014). Moreover, considering the benzimidazole resistance, the molecular study on beta-tubulin gene is highly recommended (Mohammedsalih *et al*., 2020). Today benzimidazole resistance in *Haemonchus* nematodes is highly prevalent and has been noted as a great threat to ruminant production system in many countries worldwide (Von Samson-Himmelstjerna *et al.,* 2009; Nabavi *et al*., 2011; Mohammedsalih *et al*., 2020). Sistan and Balouchestan is the widest province in southeast of Iran with very long borders with Pakistan and Afghanistan countries (Masoodian, 2003). Cattle production is prevalent especially in the north of Sistan and Balouchestan province (Nabavi, 2017). The aims of the present study were to evaluate the morphological parameters and molecular targets (ITS1-ITS2 and beta-tubulin) of cattle *Haemonchus* nematodes and to find species diversity and benzimidazole resistance.

Materials and Methods

The abomasum of 300 slaughtered cattle were inspected for the presence of *Haemonchus* nematodes. The cattle were collected from Zabol, Zahedan, and Iranshahr slaughterhouses in Sistan and Balouchestan province, southeast of Iran (100 abomasa per each district) from May 2016 to April 2017. A total of 98 adult male nematodes were identified morphologically according to the keys of Lichtenfels *et al.* (1994) and then stored in 70% ethanol until molecular analysis.

Morphological Analysis

The worms were cleared in phenol-alcohol and morphologically analyzed based on the total body length, gubernaculum length, right and left spicule length, the distance between spicule spine and the spicule posterior end (Left and right) (Achi *et al*., 2003).

Molecular Analysis

DNA Extraction and PCR

DNA was extracted from 20 male adult worms using the tissue DNA extraction kit (Takapouzist, Tehran, Iran) following the manufacturer's instructions. The extracted DNA was stored at -20°C until being used. DNA samples (ITS1, 5.8S and ITS2 regions) were amplified individually by PCR using the primer pairs described by Nabavi *et al.* (2014). For amplification of beta-tubulin fragment (including 198 and 200 codons), the primer pairs described by Nabavi *et al.* (2011) were used. PCR was performed in a total volume of 50 µL including 1x Mastermix PCR buffer (Pishgam, Tehran, Iran), 30 pmol/50 µL of each primer (Pishgam, Tehran, Iran), and approximately 2 ng per 4 µL of genomic DNA in Mastercycler® nexus (Eppendorf, Hamburg, Germany) under the following thermal pattern: 5 min incubation at 95°C to denature double-stranded DNA, 35 cycles of 45 s at 58°C (annealing step), 45 s at 72°C (extension step), and 45 s at 94°C (denaturation step). Finally, PCR was completed with an additional post amplification extension step for 10 min at 72°C. For all reactions, samples without DNA served as negative controls.

Sequencing and Data Analysis

Twenty samples (6 from Zabol, 7 from Zahedan, and 7 from Iranshahr) were selected to be sequenced

in both directions for ITS and beta-tubulin fragments. Multiple alignments of the ITS1, ITS2, and beta-tubulin sequences for each species were then used to compare and calculate similarity scores between the species. In beta-tubulin sequences, the codons No. 200 and 198 were checked for benzimidazole resistance (Nabavi *et al*., 2011). ClustalW2 sequence alignment tool (http://www.clustalw.genome.jp) was used for all alignments and calculation of similarity score. The phylogenetic trees were built using the maximum parsimony (MP) and distance methods, namely, neighbor-joining in MEGA 6.0 (Li *et al*., 2016).

Statistical Analysis

The SPSS software version 18 (SPSS Inc., Chicago, IL., USA) was used for the statistical analysis. A 95% confidence interval was calculated for mean of population.

Results

The overall infection rate was 7.33%. All of the specimens were morphologically identified as *H. contortus* (Table 1). The mean burden of *Haemonchus* nematodes was 13 per infected abomasum. DNA amplification of the ITS1-5.8s-ITS2 rDNA produced a single fragment of 975 bp (Figure 1). The length of ITS1 and ITS2 fragments was 400 bp and 231 bp, respectively. The direct sequencing of all nematodes revealed identical genotype (sequence data is available under accession number of KX829170 for 18s, ITS1, 5.8s, ITS2, and 28s). After the edition of sequences and alignment, the sequence identities ranging from 98%- 99% for ITS1 and 97%-100% for ITS2 were detected. The BLAST hit results indicated that our query ITS1 sequences were similar to the sequences of various geographical isolates of *Haemonchus* species (98-99%).

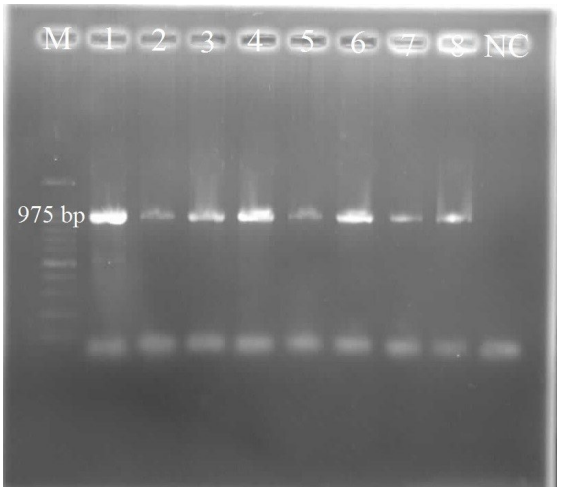

Figure 1. Amplified total fragment of *Haemonchus* ITS1-5.8S-ITS2 with 975 bp (Lanes 1-8). **M**:100bp marker, **NC**: negative control.

Table 1. Morphologic and morphometric findings of adults male *Haemonchus* nematodes from cattle of Sistan and Balouchestan province, Southeast of Iran.

	Mean body length (mm)	Spicule length (µm)	The length between right spicule barb to end (µm)	The length between left spicule barb to end (µm)	Gubernaculum length (µm)
Mean ± SE	16.85±0.31	467.53±13.6	39.83±0.96	25.66±0.62	231.59±2.26
Min & Max	15 &19	370 & 562	31 & 46	20 & 31	220 & 248
95% CI	16.19-17.50	438.83-496.22	37.80-41.86	24.34-26-97	226.82-236.37

Based on the current analysis on ITS2, the specimens had more similarity to *H.contortus*. Furthermore, the phylogenetic tree revealed close similarity between the present specimens and *H. contortus* from other parts of the world including the USA, Turkey, India, Ireland, and Iran (Figure 2).

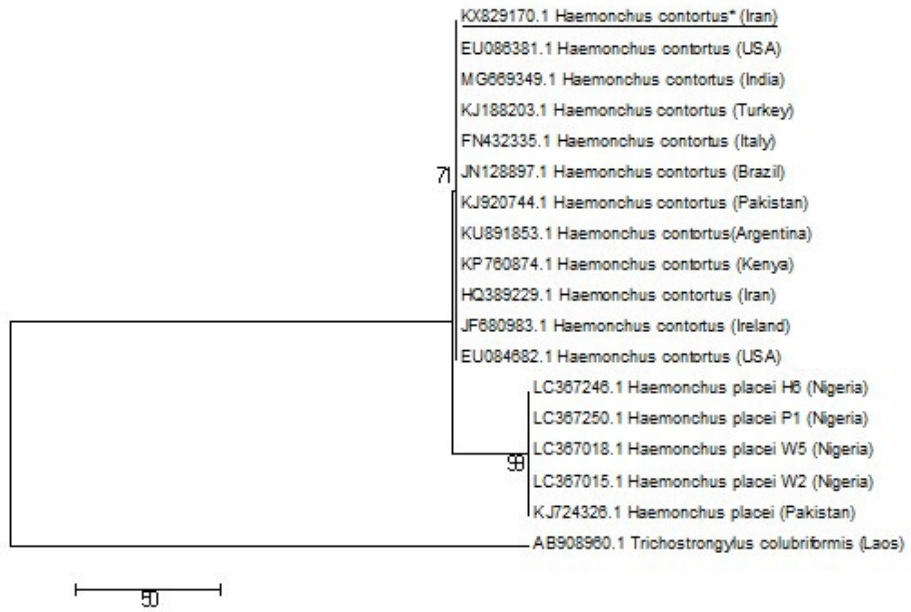

Figure 2. The phylogenetic tree of *H. contortus* based on ITS2. Molecular Phylogenetic analysis by Maximum Likelihood method. The evolutionary history was inferred by using the Maximum Likelihood method. The tree with the highest log likelihood (-600.8676) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 18 nucleotide sequences. Codon positions included were 1st+2nd+Noncoding. All positions containing gaps and missing data were eliminated. There was a total of 143 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

Amplification of the beta-tubulin fragment including 198 and 200 codons revealed the expected fragment of 403 bp in length (Figure 3). After the comparison of obtained sequences with the available data in GenBank, the specimens were found susceptible to benzimidazole with no mutation at codons 198 and 200 (Figure 4).

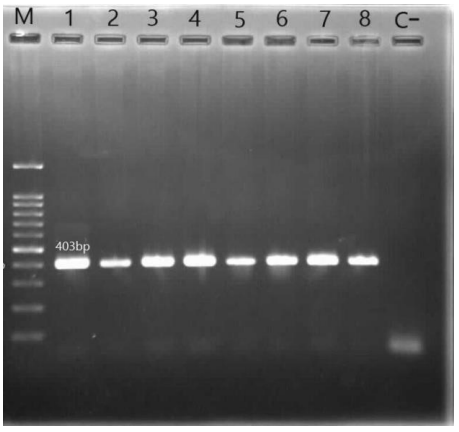

Figure 3. PCR product of *Haemonchus* Beta-tubulin gene (198 and 200 codones, Lanes 1-8). **M**:100bp marker, **C:** negative control.

Figure 4. Alignment of beta tubulin sequences (Position of codon 198 and 200) of Present study *H. contortus* with a similar isolate from GenBank (Accession number, JQ342611). Underline reveals the position of codon 198 and 200, indicating no point mutation (Benzimidazole susceptibility).

Discussion

Despite the importance of identification of *Haemonchus* species, information on its presence in cattle population of Iran is limited (Meshgi *et al*., 2015). Historically, the accurate morphological identification of *Haemonchus* species has been complicated and large numbers of specimens have presented intermediate size in body parameters like mean body length, spicule and gubernaculum length, and barb structure (Jacquiet *et al*., 1995; Rahman and Hamid, 2007; Nabavi, 2017). The morphological analysis in the present study showed similar features between the collected nematodes and *H. contortus* based on the keys of Lichtenfels *et al.* (1994). However, some species showed intermediate features common between *H. contortus* and *H. placei*. The most useful parameters in species identification were spicule lengths and distances of the barbs from the distal end. In *H. contortus*, the spicule length varied from 383 to 475 µm (mean 425 µm) and spicule barb length right/left also varied from 37 to 48 µm (Lichtenfels *et al*., 1994). In the current study, few spicule lengths were out of range. However, other parameters were in the *H. contortus* measurement ranges. Although the most common species of Haemonchus in the cattle is *H. placei,* the infection with *H. contortus* has been mostly reported around the world (Hogg *et al*., 2010; Kandil *et al*., 2018). Based on the keys of Lichtenfels *et al.* (1994), the mean body length, spicule length, and the barb structure may overlap between different species. In addition, worm body length may be influenced by nematode age and even the immunity of host (Coadwell and Ward, 1975; Nabavi, 2017; Hoglund *et al*., 2019).

Because of morphological difficulties in *Haemonchus* species identification, molecular tools were considered in the present study. Molecular analysis especially Internal Transcript Spacer 2 (ITS2) was considered as the convenient target of molecular approach in the identification of *Haemonchus* nematodes (Nabavi *et al*., 2014; Vadlejch *et al*., 2014; Meshgi *et al*., 2015; Dey *et al*., 2019; Höglund *et al*., 2019). In this study, the sequence analysis of *Haemonchus* nematodes showed no nucleotide variation in the ITS2. From the results of our molecular analysis, the collected nematodes were *H. contortus* that corroborated our morphological results. Additionally, we found some intermediate features common between *H. placei* and *H. contortus*; while in molecular analysis, they were identified as *H. contortus*. Many researchers have presented such differences in morpho-molecular evaluations of *Haemonchus* species especially in the morphology (Rahman and Hamid, 2007; Nabavi, 2017; Dey *et al*., 2019). These differences may be due to co-infections as a result of mixed grazing of small ruminants and cattle and accordingly the increased possibility of interspecies hybridization between *H. placei* and *H. contortus* in the field (Ali *et al*., 2015; Salle *et al*., 2019). Researchers believe that genetic differences in environmental tolerance arise as a consequence of a high level of polymorphism occurring in different climatic areas (Ali *et al*., 2015). Hence, *Haemonchus* species are observed in almost all regions where ruminants are raised, with the potential for outbreaks of haemonchosis, regardless of the climatic zone (Ali *et al*., 2015; Besier *et al*., 2016). In non-optimal environmental situations like Sistan and Balouchestan province of Iran, genetic diversity and morphological polymorphisms could increase in nematode populations.

Benzimidazole resistance is extremely common in *H. contortus* in small ruminants but there are only a few well-documented reports of resistance to this anthelmintic drug for *Haemonchus* nematodes infection (Mohammedsalih *et al*., 2020). It was demonstrated that single nucleotide polymorphism (SNP) was most commonly associated with resistance at codons 200 and 198 of beta-tubulin isotype1 in *Haemonchus* species (Shen *et al*., 2019; Tan *et al*., 2020). Hence the scientists used different practical and molecular methods to find such mutations in *Haemonchus* population. In this work, the specimens were susceptible to banzimidazole with no mutation. Nabavi *et al*. (2011) and Shokrani *et al.* (2012) reported similar results in *H. contortus* of small ruminants in Iran.

Conclusion

We believe that in Sistan and Balouchestan province of Iran, due to the harsh climate, ruminants' infection rate to trichostrongyloid nematodes is low (Nabavi, 2017). The prevalent species in cattle was

H. contortus with low burden. The consumption of benzimidazole compounds in treatment frequencies is significantly lower in warm rainy areas. It seems that the induction of benzimidazole resistance is less important in many parts of the world.

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Conflict of Interest

The authors declared no conflict of interest.

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مشخصات ریخت شناسی و مولکولی نماتودهای جنس همونکوس در گاوهای جنوب شرق ایران

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زمینه مطالعه: همونکوزیز یکی از آلودگی‌های مهم نماتودی در جمعیت گاوها محسوب می‌شود. برای فهم بهتر وضعیت مقاومت دارویی، همه‌گیرشناسی و همچنین راهکارهای کنترل انگل در یک منطقه جغرافیایی، تشخیص صحیح گونه‌های موجود بر مبنای ژنتیک و ریخت‌شناسی ضروری به نظر می‌رسد.

هدف: هدف از انجام این مطالعه بررسی پارامترهای ریخت‌شناسی و همچنین اهداف مولکولی (ITS1, ITS2 و ژن بتاتوبولین) در نماتودهای جنس همونکوس جداشده از گاوهای جنوب شرق ایران، به‌منظور یافتن تنوع گونه‌ای و مقاومت بنزیمیدازولی است.

روش کار: از اردیبهشت ۱۳۹۴ لغایت فروردین ۱۳۹۵، تعداد ۳۰۰ شیردان از گاوهای کشتارشده در شهرستان‌های زابل، زاهدان و ایرانشهر ارزیابی شدند. ۹۸ کرم بالغ همونکوس جدا شده، از نظر ریخت‌شناسی بررسی شدند. برای مطالعه مولکولی قطعه کلی ITS1 ، 5.8 S و ITS2 و همچنین قسمتی از ژن بتاتوبولین مورد تکثیر واقع شدند. تعداد ۲۰ کرم برای تعیین توالی قطعات ITS و همچنین بتا توبولین انتخاب گردیدند.

نتایج: از نگاه ریخت‌شناسی تمامی نمونه‌های موجود *همونکوس کونتورتوس* تشخیص داده شدند. یافته‌های تعیین توالی مولکولی ITS2 و ترسیم درخت فیلوژنی، شباهت ۹۹ درصد را بین نمونه‌های تحت بررسی و *همونکوس کونتورتوس*‌های سایر نقاط جهان نشان داد. تعیین توالی قطعه بتاتوبولین هیچگونه موتاسیونی را در کدونهای مرتبط نشان نداد و لذا تمامی نمونه‌های مورد بررسی حساس به بنزیمیدازول‌ها تشخیص داده شدند.

نتیجه‌گیری نهایی: نتایج مطالعه حاضر نشان داد که اگرچه در سایر نقاط جهان شایع‌ترین گونه همونکوس در جمعیت گاوها *همونکوس پلاسه‌ای* است؛ ولی در جنوب شرق ایران شایع‌ترین گونه *همونکوس کونتورتوس* است. همچنین القا مقاومت بنزیمیدازولی در این انگل و در این نقطه از کشور از سایر نقاط جهان پایین‌تر است.

واژه‌های کلیدی: بنزیمیدازول، ریخت‌شناسی، گاو، مولکولی، همونکوس

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