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Molecular detection of *Haemonchus contortus* in small ruminants in Basrah Province, Southern Iraq

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ABSTRACT

Background: The gastrointestinal nematode, *Haemonchus contortus* (Barber's pole worm), is a member of the Trichostrongilidae family with a major ovine parasite that resides in the abomasum of sheep, goats, and other ruminants. The spicule lengths provided the quickest and easiest character for separating most populations of *H. contortus* and *Haemonchus placei.*

Aim: Detection and diagnosis of *H. contortus* in slaughtered small ruminants from Basrah city using PCR technique with specific genes as targets: ITS2 gene (HcI) of ribosomal DNA and (Nad4) gene of mitochondrial DNA. Then, the gene sequence was used to confirm the *H. contortus* Iraqi strain.

Methods: A total of 705 slaughtered animals were examined and divided into 556 sheep and 149 goats taken from the Basrah slaughter house.

Results: The total number of infected animals with *Haemonchosis* was 90, which was divided into 78 sheep and 12 goats. The total number of isolated worms from the abomasum was 2439 for both sheep and goats, with a total infection percentage of 12.76 %, which was divided into 14.02% and 8.05% in sheep and goats, respectively. The intensity of infection was about 27.1, which was divided into 27.12 and 26.91 in sheep and goats, respectively. The identification of *Haemonchus* spp. was molecularly performed by detecting the presence of the ITS-2 (295 bp) internal transcribed spacer gene (ribosomal DNA) and the Nad4 (800 bp) nicotinamide dehydrogenase gene (mitochondrial DNA). The process included total DNA extraction and amplification of the mentioned region with specifically designed primers. The polymerase chain reaction product was sent for sequencing analysis to determine the Iraqi strain and for the phylogenetic tree. Sequencing and phylogenetic tree showed 99% identification with two American isolates (EU084691.1 and EU084688.1) for the ITS-2 gene, while the Nad4 gene showed 100% identification with one Pakistani isolate (KJ724441.1), 99% identification with two Pakistani isolates (KJ724441.1 and KJ724524.1), and 97% identification with one Pakistani isolate (KJ724524.1).

Conclusion: Barber's pole worms propagated in a large infection percentage and had an abundance of eggs that were discarded. Therefore, it forms a hazard to the animal's life, causing economic losses. Therefore, the PCR technique was a beneficial way for the genetic discovery of barber pole worms as genus *Haemonchus*, and ITS-2 and Nad4 gene were good results for the recognition of Haemonchus *contortus* as a genus and species. The isolated genes were deposited in the Gene Bank under the accession numbers LC275893.1, LC275894.1, LC275895.1, LC275896.1, LC275897.1, LC275898.1, LC275899.1, LC275900.1, and LC275901.1 as the Iraqi strain.

Keywords: *Haemonchus* spp., Basrah slaughterhouse, Polymerase chain reaction, Molecular study.

Introduction

Haemonchus contortus, commonly known as Barber's pole worm, is a Trichostrongylidae gastrointestinal nematode. It is one of the most important parasites affecting sheep, goats, and other ruminants, residing in the abomasum and following a direct life cycle (Bowman, 2009; Thamilbharathi *et al.*, 2021). Lichtenfels *et al.* (1988) noted that spicule length is a quick and reliable feature for differentiating between most populations of *H. contortus* and *H. placei.*

This parasite is highly harmful and ranks among the most significant nematodes affecting ruminants in nearly all subtropical and temperate regions worldwide (Werszko *et al.*, 2024). Mohamed *et al.* (2024) showed that the prevalence of *Haemonchus* spp. infection was 33.00%, which was one of the highest values in Upper Egypt. Clinically, the positive animals displayed symptoms such as emaciation, anemia, reduced appetite, and diarrhea.

Few studies have focused on *H. contortus* in Iraq. Faraj (2012) reported infection rates of 1.66% and

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3.84% in sheep and goats, respectively, in Basrah City. Bahidh (2011) conducted a morphological study of male and female *H. contortus* in small ruminants in Basrah Province. Al-Hasnawy (2014) noted an overall infection rate of 32.35% (66 out of 204 animals) in sheep and goats in Hilla City. Al-Diwaniyah, found that the percentage of infection with trichostyrolide was 24.16% (Jasim and A'aiz, 2021).

Molecular identification methods have also been used. Al-Zohairy *et al.* (2015) used conventional polymerase chain reaction (PCR) to amplify the ITS-2 region, producing bands at the anticipated size of 321 bp. The ITS-1 and ITS-2 regions of ribosomal DNA exist in multiple copies. Sequence uniformity among these repeats results from concerted evolution through processes such as gene duplication or unequal crossing over (Li, 1997).

Abdul Rahman *et al.* (2007) discovered six different haplotypes of ITS-2 sequences of *H. contortus* from Penang. Fentahum and Luke (2012) identified a consensus sequence length of 231 bp following ITS-2 sequence alignment and editing. Stevenson *et al.* (1995) used the ITS-2 marker to differentiate *H. placei* from *H. contortus* and found three nucleotide differences between the two species. Sequence comparisons of *H. contortus* and *H. placei* from Uzbekistan revealed six nucleotide differences (Rahman *et al.*, 2007; Fentahum and Luke, 2012).

Morphometric and molecular studies revealed no significant variation between *H. contortus* isolates from sheep and goats, that the species can infect various hosts. An Australian study found that 92 worms collected from sheep infected with cattle-originated worms included 14 adults identified as *H. contortus*, with five distinct genotypes labeled F, G, H, I, and J (Jabbar *et al.*, 2014).

This study aimed to detect and identify *H. contortus* in slaughtered small ruminants in Basrah City using polymerase chain reaction (PCR) targeting specific gene markers, namely the ITS2 gene (HcI) of ribosomal DNA and the Nad4 gene of mitochondrial DNA. These genes were used to confirm the genus and species of *H. contortus*, and the genetic identity of the Iraqi strain was verified by sequence analysis.

Materials and Methods

A random visit was conducted on the Basrah slaughterhouse in the province of Basrah to check the abomasum of slaughtered small ruminants. The organs were carefully examined for parasites, which were then isolated. The worms were washed several times with 0.9% normal saline following the procedure described by Farhan (2016). After initial identification, the worms were placed in containers containing 70% ethyl alcohol and stored at room temperature for further molecular analysis.

Genomic DNA was extracted from adult Haemonchus worms using the G-Spin Total DNA Extraction Kit (iNtRON, Korea) according to the manufacturer's instructions. The ITS-2 rDNA gene (295 bp) was amplified using primers HcI F and HcI R, while the mitochondrial Nad4 gene (800 bp) was amplified with primers Nad4 F and Nad4 R, based on methods outlined by Meshgi *et al.* (2015) and Yin *et al.* (2013) (Table 1).

PCR Amplification: DNA samples underwent PCR amplification using specific primers for the ITS-2 gene: HcI F and HcI R) and the Nad4 gene: Nad4 F and Nad4 R) to detect *Haemonchus* spp.

DNA Sequencing: PCR products were purified using the EZ-10 Spin Column DNA Cleanup Mini Preps Kit (Bio Basic Inc., Canada). The selected PCR products and primers were sent to Macrogen Inc. (USA) for sequencing. Homology analysis was performed using the BLAST tool from the National Center for Biotechnology Information, and sequence alignment was performed using BioEdit software. Five PCR-positive samples from each gene were sequenced to determine the *H. contortus* Iraqi strain. Phylogenetic analysis was conducted using the maximum parsimony method in the MEGA 5 software.

Ethical approval

Not needed for this study; however, this study was conducted in accordance with the ethical standards of the College of Veterinary Medicine, University of Basrah.

Results

Molecular diagnostics have been conducted to identify the presence of *Haemonchus* genes and to determine the specific genus and species. The extracted DNA was subjected to polymerase chain reaction (PCR) techniques to first identify the *Haemonchus* genus and subsequently to detect the ITS-2 gene (295 bp) and the Nad4 gene (800 bp). The amplification results for the ITS-2 fragment indicated that 2 sheep and 14 goats tested positive, yielding sensitivities of 6.6% and

Table 1. Sequence of primers used in the study.

Primers		Sequences	Character
ITS-2 rDNA gene	HcI-F	5′-CTC GTC TGG TTC AGG GTT-3′	*Haemonchus* Meshgi *et al.* (2015) and Yin *et al.* (2013)
	HcI-R	5′-GTA ACC TCG CTG AGC TCA-3′	
Nad4 mDNA	Nad4-F	5′-GGA TTT GGT CAG CAA ATT GAA-3′	*Haemonchus* Meshgi *et al.* (2015) and Yin *et al.* (2013)
	Nad4-R	5′-GCC TGC AAA TGA ATT AAC A-3′	

70% for sheep and goats, respectively. In contrast, the amplification results for the Nad4 gene revealed that 14 sheep and 11 goats were positive, with sensitivities of 46% and 55%, respectively.

The results of the PCR indicated the amplification of both the ITS-2 and Nad4 genes from the contortus species. Four samples were used for sequencing the ITS-2 gene, and five samples were used for Nad4 gene sequencing. The alignment of our sequences against the copies of the ITS-2 and Nad4 genes available in the NCBI database demonstrated a high degree of compatibility, with percentages of 99% and 100%, respectively (Table 2). Furthermore, a comparison between the recent findings of the ITS-2 gene sequence (designated as HcI) and the Gene Bank database reveals a notable 99% similarity with two American variants, listed under the accession numbers EU084691.1 and EU084688.1.

Conversely, the analysis of the Nad4 gene sequence results in relation to the GenBank database indicated a complete match of 100% with one Pakistani sample (KJ724441.1). Furthermore, there was a 99% similarity with two additional Pakistani samples (KJ724441.1 and KJ724524.1), while a 97% match was observed with another Pakistani sample (KJ724524.1), as detailed in Table 3.

The sequences pertaining to the current investigation have been deposited in the Gene Bank under the accession numbers LC275896.1, LC275895.1, LC275894.1, and LC275893.1 for the ITS2 gene and LC275901.1, LC275900.1, LC275899.1, LC275898.1, and LC275897.1 for the Nad4 gene as representatives from Iraq. In this study, phylogenetic trees were constructed based on the comparative analysis of these sequences alongside those of other *H. contortus* strains published in the Gene Bank. The phylogenetic analysis was conducted using MEGA software, illustrating the evolutionary relationships among the four isolates of *H. contortus* identified in this study and additional strains available in the Gene Bank, utilizing the partial nucleotide sequences of the ITS-2 rDNA gene (Table 4).

The phylogenetic relationship among the five *H. contortus* samples obtained during the recent study and other *H. contortus* strains from the Gene Bank was assessed based on the partial nucleotide sequence of the Nad4 mitochondrial DNA gene (Table 5).

Analysis of the ITS-2 gene reveals that the phylogenetic relationship among the Iraqi isolates exhibits a 99% identity with an American isolate. The observed variation is attributed to a single-nucleotide difference between the two American isolates (EU08469.1 and EU084688.1). In contrast, the assessment of the Nad4

Table 2. ITS-2 (HcI) gene sequences production significant alignments For H. contortus.

No. of Sample	Score	Expect	Identities	Gaps	Strand
HcI-1	409 bits (221)	2e-110	224/225 (99%)	1/225 (0%)	Plus/ Plus
HcI-2	409 bits (221)	2e-110	224/225 (99%)	1/225 (0%)	Plus/ Plus
HcI-3	405 bits (219)	2e- 109	224/ 226 (99%)	2/ 226 (0%)	Plus/ Plus
HcI-4	411 bits (222)	5e-111	229/ 232 (99%)	2/232 (0%)	Plus/ Plus

Table 3. Nad4 gene sequences production significant alignments For H. contortus.

No. of Sample	Score	Expect	Identities	Gaps	Strand
Nad4-1	427 bits (231)	5e- 116	235/ 237 (99%)	0/ 237 (0%)	Plus/ Plus
Nad4-2	726 bits (393)	0.0	401/ 405 (99%)	0/ 405 (0%)	Plus/ Plus
Nad4-3	401 bits (217)	3e- 108	224/ 227 (99%)	2/ 227 (0%)	Plus/ Plus
Nad4-4	331 bits (179)	3e- 87	179/ 179 (100%)	0/179 (0%)	Plus/ Plus
Nad4-5	464 bits (251)	8e- 127	268/ 276 (97%)	2/ 276 (0%)	Plus/ Plus

Table 4. Phylogenetic Relationship among Haemonchuscontortus Strains for ITS-2 Gene.

Sample name	Similar with	Identity %	Alignment matching
1_HCIF	EU084691.1	99	639–862
2_HCIF	EU084688.1	99	639–863
4_HCIF	EU084688.1	99	639–863
5_HCIF	EU084691.1	99	632–862

Table 5. Phylogenetic relationship among Haemonchuscontortus Strains for Nad4Gene.

Sample name	Similar with	Identity %	Alignment matching
1_Nad4F	KJ724441.1	99	1–237
2_Nad4F	KJ724524.1	99	1–405
3_Nad4F	KJ724524.1	99	1–225
4_Nad4F	KJ724441.1	100	1–179
5_Nad4F	KJ724524.1	97	1–274

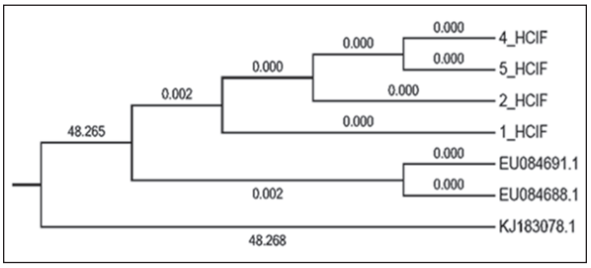

Fig. 1. UPGMA phylogenetic tree based on partial nucleotide sequence of the ITS-2 rDNA genes of *H. contortus*.

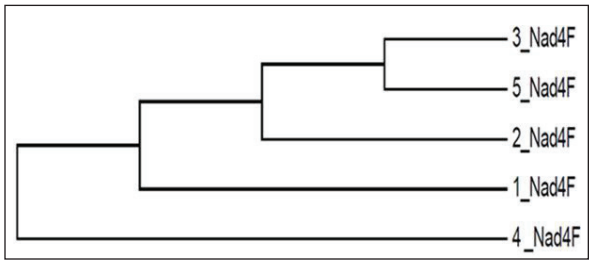

Fig. 2. Neighbor-joining phylogenetic tree based on the partial nucleotide sequence of the *H. contortus* Nad4 mDNA genes.

gene indicates that Iraqi samples 3 and 5 share complete identity (100%) and demonstrate considerable similarity to other samples (2, 4, and 1). Notably, variations exist, with one nucleotide difference observed compared to two Pakistani isolates (HJ7244441.1 and KJ724524.1) and a divergence of three nucleotides with another Pakistani isolate (KJ724524.1) (refer to Figs. 1 and 2).

Discussion

Molecular identification of *H. contortus* is essential for understanding genetic variation and aiding surveillance and control strategies. The PCR results in this study are consistent with those of Meshgi *et al.* (2015), who identified *H. contortus* using ITS-2 gene amplification (295 bp) in both sheep and goats.

Some findings differ from those of Mangkit *et al.* (2014) in Thailand, Yin *et al.* (2013) in China, Abramatov *et al.* (2013) in Uzbekistan, Al-Zohairy *et al.* (2015) in Egypt, and Thamilbharathi *et al.* (2021) in Kerala, who reported a 321-bp ITS-2 PCR product containing 231 bp of core sequence and 90 bp of flanking regions. Santos *et al.* (2014) detected two bands (1.32 kb and 1.63 kb) in male *H. contortus* through PCR, whereas Gharamah *et al.* (2012) reported the Nad4 gene to be approximately 600 bp long with no coding region amplification. These differences likely stem from geographical and environmental factors influencing the genetic and morphological traits of the parasite.

The ITS-2 and Nad4 genes of *H. contortus* were sequenced for the first time in Iraq. The ITS-2 gene sequence matched 99% with a Florida isolate (Garretson *et al.*, 2009), whereas the Nad4 sequences revealed 100% identity with a Pakistani isolate (document_number_3.1) and 99%–97% identity with others reported by Hussain *et al.* (2014). Hussain documented significant Nad4 gene diversity, noting 177 polymorphic loci among *H. contortus*, *H. placei*, and hybrids, including 113 sites informative for parsimony. Chinese isolates (Yin *et al.*, 2013) exhibited 2.6% sequence variation in ITS-2 among 152 individuals, with Nad4 nucleotide diversity ranging from 0.018 to 0.037. Gharamah *et al.* (2012) found Nad4 nucleotide diversity of 0.032–0.044 in Malaysia and 0.021–0.036 in Yemen.

The phylogenetic analysis in this study supports the hypothesis that the same *H. contortus* strain can infect sheep and goats. For example, ITS-2 samples HcI4 and HcI5 (from goats) matched 100% with sheep isolates. Similarly, Nad4 samples 3 and 5 (from sheep) were genetically identical to samples 1, 2, and 4 (from goats), reviewing that the strains were uniform across different host species**.**

Conclusion

Polymerase chain reaction (PCR) is a reliable and efficient method for identifying the genetics of *H.* contortus. The ITS-2 and Nad4 genes were effective markers for confirming the identification of species. The gene sequences from this study have been deposited in GenBank under the accession numbers:

ITS-2 gene: document_number_5.1–document_number_8.1

Nad4 gene: document_number_9.1–document_number_13.1

These sequences represent the first molecular characterization of the Iraqi strain of *H. contortus.*

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Author's contributions

The second author conceived the study design; the first author performed the laboratory work. Both authors have read and approved the final version of the manuscript.

Conflict of interest

The authors declare no conflict of interest.

Data availability

All the information supporting the discoveries in this regard is accessible in the manuscript.

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