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Research Article

Genetic Characterization of *Cysticercus tenuicollis* Isolated from Sheep Using PCR and Sequencing

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Abstract | *Cysticercus tenuicollis*, the larval stage of *Taenia hydatigena,* is a parasitic organism that significantly affects various intermediate hosts, particularly sheep. This study aimed to genetically characterize *C. tenuicollis* isolates from naturally infected sheep in Maysan Province Southern Iraq, by PCR and sequencing of the mitochondrial 12S rRNA gene fragment. Sixty larval stage isolates were obtained from male sheep aged 1–2 years, and genomic DNA was extracted for molecular analyses. PCR amplification using specific primers targeting the 12S rRNA gene yielded positive results for all the samples confirming a 100%. DNA sequencing and BLAST analysis revealed high genetic similarity (98–100%) to previously recorded *C. tenuicollis* isolates particularly from Iraq, Iran, and Turkey. Sixteen new isolates were successfully registered in the GenBank database. Phylogenetic study with MEGA software showed that local isolates belong to the same clade as *C. tenuicollis* isolates from neighboring regions, with minimal nucleotide variants that indicate a local evolutionary divergence. Eight nucleic acid variations were detected compared with the reference sequences with most samples exhibiting three specific variations. The phylogenetic tree based on 12S rRNA sequences demonstrated the presence of only one species *T. hydatigena*, the with sequences clustered into adjacent phylogenetic branches. The genetic diversity observed within the samples studied might be related to the detected variations and the potential for transmission across intermediate hosts, thereby enhancing genetic variation among parasite populations worldwide. These results enhance the understanding of local strain diversity and phylogenetic relationships in targeted parasite control strategies within Iraq and its neighboring areas.

Keywords | *Cysticercus tenuicollis*, 12SrRNA, DNA sequence, Phylogenetic tree, Sheep, *Taenia hydatigena*

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INTRODUCTION

Cysticercus tenuicollis is the larval stage of *Taenia hydatigena*, and is a parasitic organism that significantly affects various intermediate hosts, including ruminants particularly sheep and goats. Of great concern to veterinary and public health is the fact that this organism may lead to economic losses in livestock production and also it has zoonotic potential. It has been reported that the parasitic infection is quite common in the whole variety of geographical sites, yet the prevalence of the infection is considerably high in Europe, Asia, and Africa, which is explained by the adaptive capacity to various places and various hosts (Luo *et al*., 2017; Kılınç *et al*., 2019). The life cycle of *Cysticercus tenuicollis* begins with the ingested by the feces of infected definitive hosts including dogs after being consumed by intermediate hosts like sheep cysticerci further develop into eggs capable of infecting other visceral organs although the liver and peritoneum are most affected. This transition emphasizes environmental and dietary

factors contribute to the transmission cycle and in many cases this process is a major cause of health consequences in the affected livestock given that the carriers can be asymptomatic and these creatures can be present in herds (Hama *et al*., 2018; Mohammed, 2020).

The accurate identification of *T. hydatigena* in geographically diverse areas is essential with the help of molecular tools. As an example, the development of mitochondrial DNA sequencing has made it possible to define haplotypes and genetic variations in different hosts with more accuracy and study the evolutionary patterns of this parasite and its distribution in Central and Eastern Europe better (Ohiolei et al., 2021; Sgroi *et al*., 2020).

In Iraq, only a few molecular studies have been conducted in the North and South provinces, and no complete genetic information can be found on the Maysan Province which is an agriculturally important area with a high population of small ruminants. This study sought to fill this gap by genetically characterizing *Cysticercus tenuicollis* isolates of naturally infected sheep of Maysan province through PCR and sequencing of mitochondrial 12S rRNA gene. These findings sought to give novel understanding of the diversity of local strain, as well as add to phylogenetic knowledge, and inform parasite control efforts, especially in Iraq and the adjacent areas.

MATERIALS AND METHODS

Study design and location

This molecular descriptive study was conducted in Maysan Province, southern Iraq between May and June 2025. Samples were collected from sheep slaughterhouses located in the city center and surrounding districts of the province.

Sample collection and preparation

Sixty larval stage (*Cysticercus tenuicollis*) isolates were obtained from naturally infected male sheep (aged 1–2 years) slaughtered in local abattoirs in Maysan Province. During the post-mortem inspection, cysts were collected from the omentum and peritoneal cavity. The collected cysts were immediately transported to the laboratory under sterile conditions for molecular analysis. For molecular experiments, the collected cysts were meticulously washed with sterile saline and stored in 70% ethyl alcohol until DNA extraction.

Genetic analysis (PCR)

Genomic DNA was extracted using a commercial extraction kit (Geneaid, Korea) according to the manufacturer's instructions. Specific primers targeting the *12SrRNA* gene were as follows: Forward, 5'-AGGGGATAGGACACAGTGCCAGC-3' and reverse: 5'- CGGTGTGTACATGAGCTAAAC - 3' (Rostami *et al*., 2015) were used for amplification. PCR has been run on a thermal cycler following the following protocol: first 94C denaturation of the sample at 94C (3 min), then 30 cycles of 94C denaturation at 60s, 56C annealing at 90s, and 72C extension at 60s, and lastly 10 minutes with the same denaturation temperature at the end of 30 cycles (Rostami *et al*., 2015). Electrophoresis of the PCR products was done through 2% agarose gel, and the appearance of a 489bp band was taken to be positive about the *12SrRNA* gene.

Sequencing analysis

After validation of the target amplicon on electrophoresis, the PCR products were sent to Macrogen corporation in South Korea lab where they were sequenced. Analysis of the obtained sequences was performed with the help of the BioEdit software and sequence alignment with the GenBank database entries was performed with the help of the BLAST tool. All the identified variants in the *T. hydatigena* gene were annotated with the Snap Gene Viewer, ver. 4.0.4 (https://www.snapg ene.com).

Statistical analysis

To identify the distribution of the isolates, a descriptive analysis was performed, and the level of genetic similarity was calculated by means of MEGA software (version 11). Calculation of genetic distances and a phylogenetic tree was deduced using neighbor-joining (NJ) technique based on NCBI-BLAST server (Zhang *et al*. 2000). A tree with the identified variant plus all the others found was constructed with the neighbor-joining algorithm and was visualized with the iTOL suite to produce a conventional tool to be used in constructing a clade (Letunic and Bork, 2019). Each of the classified phylogenetic species groups present in the phylogenetic tree was annotated with the sequences. Sixteen of the representative isolates were deposited in GenBank, and accession numbers were provided.

Ethical considerations

All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the College of Pharmacy, University of Misan, and were conducted in accordance with national ethical guidelines to ensure the welfare of the animals.

RESULTS

The study results showed that all larval stages of *Taenia hydatigena* isolated from

the sheep are from the peritoneal cavity and omentum (Figure 1A, B). Using *12S rRNA* gene primers the results of the current study showed a clear gene band of the expected size (approximately 489 bp) in all studied samples,

confirming the molecular diagnosis of *C. tenuicollis* infection in slaughtered sheep in Maysan Province. Sixty samples yielded positive results representing 100%. PCR results were confirmed by agarose gel electrophoresis, where bands appeared at specified locations. The Figure 2 showed the genomic DNA of *Taenia hydatigena*. As shown in Figure 3, the molecular size of *12SrRNA* gene was 489bp.

Figure 1: A and B larval stage of *Taenia hydatigena* (*Cysticercus tenuicollis*).

DNA SEQUENCING IN *CYSTICERCUS TINUCOLICES* OF *T. HYDATIGENA*

PCR-positive samples were subjected to genetic sequencing, and BLAST analysis revealed a high level of genetic similarity with previously recorded *Cysticercus tenuicollis* isolates in the GenBank database, ranging from 98% to 100%, particularly with isolates from Iraq, Iran, and Turkey.

Sixteen new isolates were successfully registered in GenBank under the following accession numbers: LC885134, LC885135, LC885136, LC885137, LC885138, LC885139, LC885140, LC885141, LC885142, LC885143, LC885144, LC885145, LC885146, LC885147, LC885148, and LC885149.

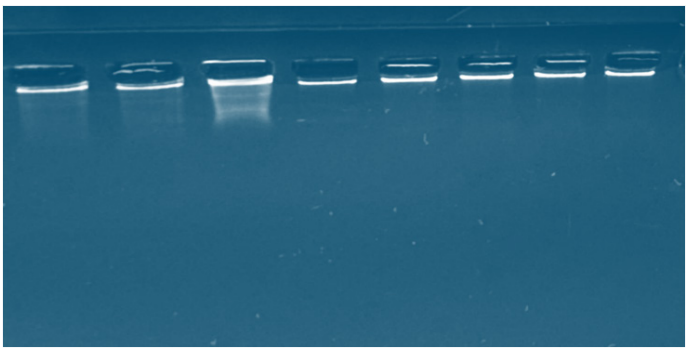

Figure 2: Genomic DNA of *Cysticercus tinucolices.*

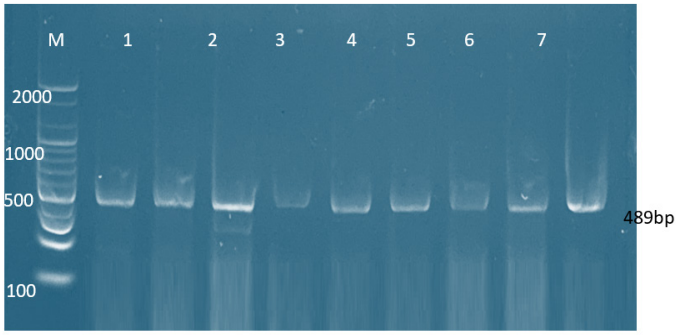

Figure 3: Agarose gel electrophoresis of *12SrRNA* gene amplification; M: ladder, 1-9: positive results. (1.5% agarose, 70 v).

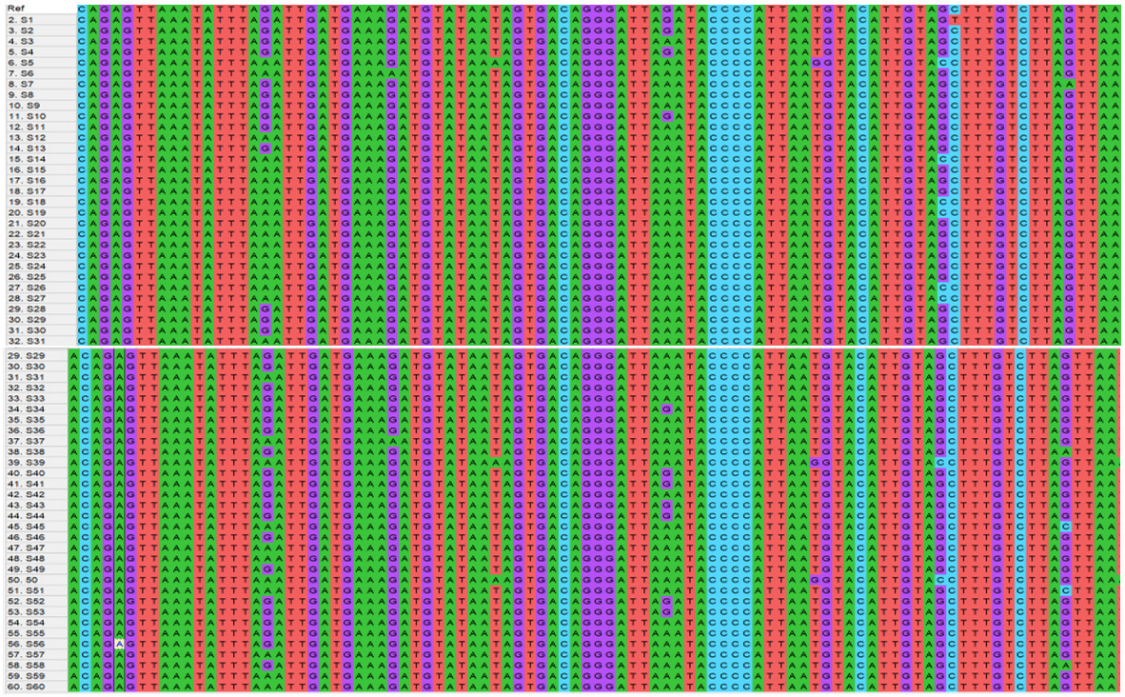

Figure 4: Nucleic acid sequence alignment of 60 samples with their corresponding reference sequences of the *12SrRNA* gene within the *T. hydatigena* genomic DNA sequences. The symbol "ref" refers to the NCBI reference sequences, and S refers to the sample.

Phylogenetic analysis performed using MEGA software showed that the local isolates clustered within the same clade as *C. tenuicollis* isolates from neighboring countries, with some minor nucleotide variations, which may indicate limited local evolutionary divergence.

In addition, the alignment results of the 489bp samples revealed eight nucleic acid variations compared with the corresponding *T. hydatigena* reference sequences (Figure 4). The sequences were prepared by aligning the samples investigated with the most relevant sequences deposited in the NCBI database.

To summarize all the results obtained from the sequenced 489bp fragments, the exact positions of the observed variations are described in Table 1. Most of the studied samples were exposed to three variations (G127A, G162A, and G186C) at positions (127, 162, and 186), respectively. Samples (2, 4, and 10), which did not exhibit any variation, were detected in the other studied samples. Samples (5, 39, and 50) with the greatest variation were (G127A, T146A, G162A, T175G, and G186C).

Table 1: The pattern of the observed variations in the 489bp of the *12SrRNA* amplicons in comparison with the NCBI reference sequences.

Position in the PCR fragment	Variant	Sample
127	G127A	5,6,12,14-27,30,37,39,45,47-48,50-51,57,59-60
138	G138A	6,37
146	T146A	5,39,50
162	G162A	3,5-9, 11-33, 35-39,42,45-51,54-60
175	T175G	5,39,50
186	G186C	5,14,18,19,26,27,39,50
197	G197A	7,38,58
197	G197C	45,51

A phylogenetic tree was generated based on the investigated *12SrRNA* nucleic acid sequences in the parasitic samples. Along with the other deposited DNA sequences, this phylogenetic tree contained the currently investigated samples (1-60) aligned with highly related sequences in Tamura-Nei mode. In the constructed tree, the total number of aligned nucleic acid sequences was 60. This tree included only one species, *T. hydatigena*, which represented the only incorporated nucleic acid sequence within the tree. Based on the analyzed genetic sequences of *T. hydatigena,* the analyzed *12SrRNA* sequences clustered into several adjacent phylogenetic branches Figure 5. In this phylogenetic tree, the samples (6 and 37) were closely related to the reference sequence, and those samples were related to adjacent branches with samples (50, 5 and 39) and last sample (5, 39, 50) harbored most of the genetic variations listed in Table 1.

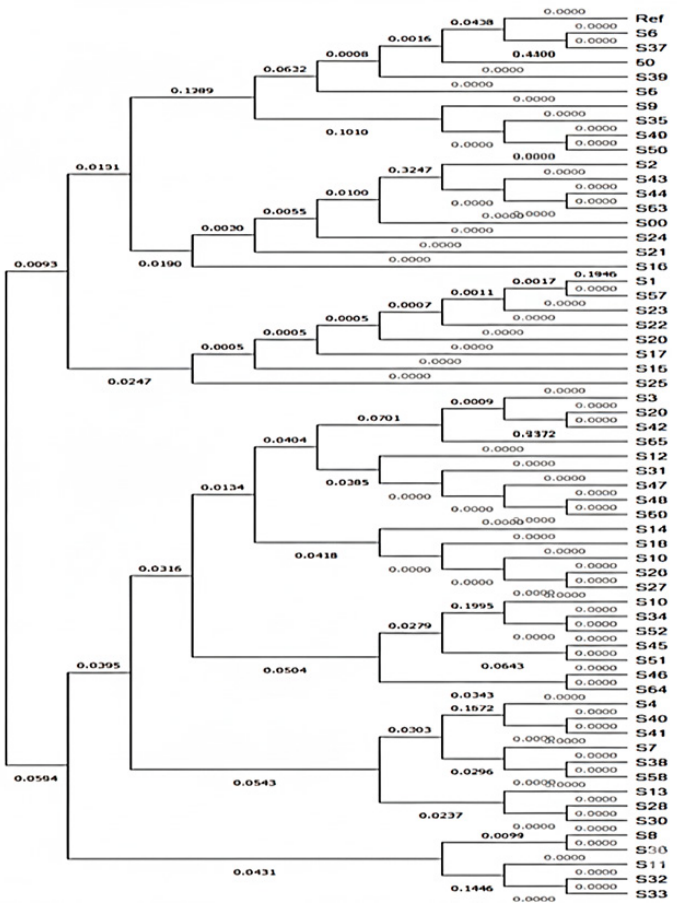

Figure 5: The evolutionary history was inferred using the neighbor-joining method (Saitou and Nei, 1987). The optimal tree is shown (next to the branches). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004) and are expressed in terms of the number of base substitutions per site. This analysis included 60 nucleotide sequences. The codon positions included were 1st+2nd+3 rd, and all ambiguous positions were removed for each sequence pair (pairwise deletion option). The final dataset contained 2632 positions. Evolutionary analyses were conducted using MEGA11 (Tamura *et al.*, 2021).

The total number of aligned nucleic acid sequences in the constructed tree was 78. The phylogenetic tree is shown in Figure 6 show that all studied samples belonged to (*T. hydatigena*), which represented the only incorporated nucleic acid sequences within this tree. Based on the analyzed genetic sequences of *T. hydatigena,* the analyzed *12SrRNA* sequences were clustered into many adjacent phylogenetic branches, which indicated a similarity of this organism with the analyzed *12SrRNA* sequences.

Sample (S1) was related to KU671395.1 of *Taenia hydatigena*, Egypt, which was separated from other branches (LC749828.1). Iraq, Mosul, KX094340.1 *Taenia*

hydatigena. Iran; KX084714. Iran; PV389993.1. China). From this phylogenetic tree, the samples (39 and 50) were closely related in a separate branch with the sequence of sample (5), and these samples were related to adjacent branches with other samples (6 and 37), (57 and 60), (24, 23, 20, 22, 17, 16, and 15), which samples were closely related to adjacent branches with *T. hydatigena* isolates in the local isolates (LC749828.1. Iraq, Mosul) and adjacent countries such as Iran and China.

The samples (34 and 52) were bound to samples (10,53,2,44) by adjacent branches, which were related to *Taenia hydatigena* isolates from Iraq (LC746809.1 *Taenia hydatigena*, LC746826.1. Iraq, Mosul, MK858233.1, MK858249.1, LC749827.1. Iraq, Mosul); and from a neighboring country (Iran): KU749826.1 *Taenia hydatigena*, KU750812.1., KU745527.1, KX094336.1); or from Egypt: KU671388.1., KU671390.1., KU671391.1, KU671392.1; KX671395.1, KX671392.1, KX671391., KX671388.1, KX671390.1) and India (LC749826.1 *Taenia hydatigena)*.

Figure 6: The evolutionary history was inferred using the neighbor-joining method (Saitou and Nei, 1987). The optimal tree is shown. (next to the branches). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al*., 2004) and are expressed in terms of the number of base substitutions per site. This analysis involved 78 nucleotide sequences. The codon positions included were 1st+2nd+3rd, and all ambiguous positions were removed for each sequence pair (pairwise deletion option). The final dataset contained 191 positions. Evolutionary analyses were conducted using MEGA11 (Tamura *et al*., 2021).

DISCUSSION

This study provides the first comprehensive genetic characterization of *Cysticercus tenuicollis* isolates from sheep in Maysan Province, southern Iraq, based on 12S rRNA gene sequencing. All isolates showed very high similarity (98–100%) with reference sequences from Iraq, Iran, and Turkey, supporting the existence of a genetically conserved *Taenia hydatigena* population across the Middle East. Such homogeneity has been reported previously in northern Iraq, Egypt, and Iran, reflecting the conserved nature of the 12S rRNA marker and the possible influence of regional livestock trade and the movement of definitive hosts such as dogs.

Although overall diversity was limited, the identification of eight nucleotide variations, particularly the recurrent SNP triad (G127A, G162A, G186C), provides evidence of local micro-evolutionary divergence. These SNPs could serve as useful genetic markers for rapid molecular screening in epidemiological surveys. The presence of highly conserved haplotypes combined with a few polymorphic variants is consistent with the pattern of high haplotype diversity, but low nucleotide diversity is often observed in parasitic cestodes with widespread distribution. This may indicate recent population expansion from a common ancestor and ongoing gene flow facilitated by animal movement across borders.

Its use of a single mitochondrial marker (12S rRNA) is also a weakness since it is a strong species confirmation but can underestimate finer scale genetic variance. It would be helpful to use rapid-evolving markers like cox1 or nad1 in future studies to reveal concealed population structures and the geographic origins. In addition, a broader sampling to various hosts (e.g, dogs, goats) and other provinces of Iraq would be useful in trying to understand the dynamics of the transmission of the parasite. Moreover, the rapidly added sequences in GenBank increase the reference database that is used in designing other molecular diagnostic tools like multiplex PCR assay to detect taeniid infections simultaneously.

Intraregional divergence in 12S rRNA genes was also reported, with low intra-regional divergence of the molecule reported in both the northern part of Iraq and the isolates had a close relatedness with those of Iran (Mohammed, 2020). In the same way, as shown in analyses of livestock isolate in Iran (Sarvi *et al*., 2020) and sheep in Egypt (Abbas *et al*., 2021), high sequence identity and low nucleotide diversity at a single locus indicated a widespread and genetically homogenous population of parasites. The 12S rRNA marker is not special since this pattern of limited divergence has been observed in other mitochondrial genes in Sudan (Muku *et al*., 2020) and Central Europe (Jarošova *et al*., 2022) as well. This is however contrary to results of other studies which utilized more variable mitochondrial markers. A study done in Turkey based on nad1 gene indicated a significantly larger number of haplotypes in both sheep and goats (Kilinc *et al*., 2019) which indicates that there is higher genetic diversity than what the more conservative *12S rRNA* gene can capture. Moreover, a multi-regional study based on PCR-RFLP named two different mitochondrial lineages with the geographically structured (Ohiole *et al*., 2022) degree of complexity which could not be seen in our single-locus study. This difference can probably be explained by the fact that the rate of evolution of mitochondrial genes is varying; ribosomal RNA genes as 12S rRNA are functionally constrained and evolve more slowly than protein-coding genes such as cox1 or nad1, which are great in terms of confirming species, but less effective in terms of dissecting fine-scale population structure. The homogeneity that we could have observed in our study could hence be due to the conserved nature of the marker we chose and also because the region might be experiencing a lot of gene flow due to the livestock trade and the migration of the definitive hosts.

In addition to this general species-level grouping we found a set of eight nucleotide variants in the 489 bp 12S rRNA fragment with a common triad of SNPs (G127A, G162A, G186C) found in most isolates. This observation of the few common polymorphisms is consistent with the low nucleotide diversity observed in other 12S rRNA studies of *T. hydatigena* in Iraq (Mohammed, 2020), Iran (Sarvi et al., 2020), and Egypt (Abbas *et al*., 2021) that are low in nucleotide diversity and high in haplotype diversity usually indicating a recent population explosion of a common ancestor. Contrarily, other studies have found a significantly higher amount of genetic variation. As an illustration, one comprehensive study found 39 different haplotypes of 12S rRNA with a pairwise variation of up to 2.1 (Rostami *et al*., 2015), and another study conducted in Turkey found 34 different haplotypes using the cox1 gene (Karakoc *et al*., 2024). Such differences can be due to the differences in the fragments of the genes studied, geographical space of the study, or different populations of hosts. Although the SNP panel we have is limited to estimate the underlying genetic diversity, the repetitive count of variants found presents us with a viable instrument. Such SNP triad may be used as an easy and affordable preliminary haplotyping marker in regional surveillance schemes to enable rapid screening to be done, without necessarily the full sequence.

The samples (5, 39 and 50) were closely related with the other samples (6, 37, 57 and 60) in close branches with other samples and isolates (local and adjacent country): Iran, Egypt, and other countries as: India.

The current findings were consistent with findings by locally study in Mosul city by Alhankawe *et al*. (2025) documented that *T. hydatigena* isolates were more related with *T. hydatigena* in Sulaymaniyah-Iraq and Iran. This could be because the Sulaymaniyah province and Iran which are close to the Mosul city have the same temperatures and animal habitats. Thus, it will be necessary to understand that the genetic identification of the parasite will be the key to controlling this parasitic disease (Wang *et al*., 2018).The close relatedness between the samples under investigation and locally isolated ones as MK858233.1.1, and MK858249.1.1 Iraq, and *T. hydatigena* isolates of Iran as: KU745527.1, and KX094336.1 is possibly connected with the animal trade at the neighboring country to the Kurdistan region and at that region to the rest of the governorates of Iraq, including Baghdad (Al-Sudani and Al-Amery, 2022).Huge domestic animal migration into Kurdistan-Iraq came into the country in the form of imports both in Turkey and Iran (Hama *et al*., 2018).

The extensive phylogenetic studies, which incorporated high numbers of region-specific reference sequences, strongly supported the conclusion that all the larval cysts in this study were *T. hydatigena* which had been taken out of sheep. This observation supports the extreme specificity of this parasite to its intermediate host and the tissue tropism resulting in *C. tenuicollis* formation. It is in line with the multiple abattoir-based molecular surveys in the Middle East and other countries where sheep cysticerci are always found to be *T. hydatigena* (Mohammed, 2020; Sarvi *et al*., 2020; Wakid and Alsulami, 2022). Although our sample of intermediate hosts produced only one species, in cases where definitive hosts are studied the situation is usually much more complicated. As an instance, canid feces molecular analysis has identified co-circulation of several species of the Taeniidae group, such as *T. hydatigena* and others (Ulziijarga *et al*., 2020; Mirbadie *et al*., 2019). This reveals one of the major differences: Whereas dogs in the area might harbor a varied population of tapeworms, the pathology of cysticercosis in sheep viscera seems to be brought about by *T. hydatigena* alone. This explains the goal of control interventions to minimize the losses in the economic production of sheep. The application of the *12S rRNA* gene in precise species-level diagnosis is also well established and supported additionally by its application in the development of diagnostic tests, such as multiplex PCR assays to distinguish between the common taeniid species (Zhu *et al*., 2019).

Demonstration of a genetically homogeneous *T. hydatigena* population in circulation in southern Iraq and other adjacent countries has important implications to veterinary public health and disease control. The parasite causes considerable economic losses through the condemnation of offal and reduced livestock productivity (Munoz-Guzman *et al*., 2023). Our findings strongly support the implementation of coordinated, cross-border control strategies, as interventions in one country will likely be impacted by parasite populations in adjacent regions. Control efforts should prioritize the definitive host. This includes regular anthelmintic treatment of shepherd, stray, and domestic dogs with praziquantel, alongside improved public education and enforcement of policies to prevent dogs from accessing raw offal at abattoirs and on farms (Khaled *et al*., 2020; Zheng, 2016). Furthermore, our work demonstrates the value of integrating molecular typing into routine abattoir surveillance. The validated 12S rRNA marker and the defined SNP panel can be used for rapid confirmation and basic tracking of parasite lineages, while the 16 new sequences we have deposited in GenBank enhance the regional reference database for future phylogeographic studies.

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NOVELTY STATEMENT

The study successfully registered sixteen new isolates in the GenBank database under accession numbers LC885134 through LC885149, providing new genetic reference data for future research. This represents a significant contribution to the global genetic database for this parasite species also the research identified eight specific nucleotide variations in the 12S rRNA gene fragment, with a characteristic triad of single nucleotide polymorphisms (SNPs) - G127A, G162A, and G186C - present in most local isolates. This SNP pattern could serve as a molecular marker for regional parasite populations.

AUTHORS CONTRIBUTION

ES: Conceptualization, experiment design, supervision, manuscript drafting, correspondence, sampling, data analysis, writing and revision. HAHK: Literature review, figure preparation, referencing, statistical analysis, discussion enhancement, and formatting.

Generative AI and AI-assisted technology statement

The authors declare that no Genrative AI was used in the

creation of this manuscript.

Conflict of interest

The authors have declared no conflict of interest.

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