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J Vet Res 64, 275-280, 2020
DOI: 10.2478/jvetres-2020-0034

Molecular study of *Cysticercus tenuicollis* from slaughtered sheep in Sulaymaniyah province, Iraq

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Received: September 7, 2019 Accepted: May 4, 2020

Abstract

Introduction: Cysticercosis caused by the larval stage of *Taenia hydatigena* is economically the most important endemic parasitic disease in Iraq. Few data are available relating to the genetic divergence of this helminth. This study aimed to molecularly characterise *Cysticercus tenuicollis* isolates from sheep in Sulaymaniyah province, Iraq. **Material and Methods:** DNA extraction and amplification of specimens of *C. tenuicollis* from 46 sheep were conducted by PCR for the mitochondrial 12S rRNA gene. The 19 amplicons were subjected to purification and partial sequencing. **Results:** Five 12S rRNA nucleotide sequence haplotypes were found. The pairwise nucleotide difference between haplotypes of 12S rRNA gene ranged from 0.2% to 0.7%. Four out of the five haplotypes of *C. tenuicollis* contained one to two base mutations and were discovered in Iraq for the first time, and this may be a unique mutation globally which has not been recorded previously. Three newly recorded haplotypes contained only one single mutation, and the other one contained two mutations. Phylogenetic analysis showed that all isolated strains were closely related to Iranian sheep isolates. **Conclusions:** Four new strains of *T. hydatigena* were discovered for the first time in the study area.

Keywords: sheep, *Cysticercus tenuicollis*, genetic variation, phylogeny, Iraq.

Introduction

The cyclophyllidean cestodes include the most significant members of the *Taenia* genus. Species belonging to this genus are responsible for significant economic losses and harm to health in humans and livestock. *Taenia hydatigena*, which was discovered by Pallas (1766), is a universal and widely distributed helminth infesting carnivores as a definitive host and with a metacestode, *Cysticercus tenuicollis*, which can infect herbivores (23). Canids release gravid segments in the faeces, and the eggs, which are scattered in the natural environment are then incidentally ingested by ruminant secondary hosts. The discharged oncospheres relocate to the liver by means of the circulatory system and cause haemorrhagic and fibrotic lesions known as hepatitis cysticercosa (10). This condition may either result in the death of animals at young ages because of serious fibrous hepatitis or the condemnation of the liver after slaughter (8).

A precise characterisation of the causal agents of various *Taenia* infections is crucial for understanding the transmission dynamics and epidemiology, and for vaccine development, definitive diagnosis, treatment, and useful control and preventive measures of the taeniid species. Comparative genetic analyses give reliable differentiation of genetic variants and improve our understanding of the character and significance (including for animal and public health) of intra-specific difference. Deoxyribonucleic acid sequence analysis of conservative genes could be a sensitive and reliable tool for estimating genetic connections between totally different helminth species. Mitochondrial genes are amongst the most popular markers for molecular-based approaches to ecology, population genetics and evolutionary biology and have been popular targets for molecular-based methods of species identification (4, 6, 22).

The molecular study of *T. hydatigena* isolates has been conducted in various locations around the world

(11, 13, 17, 24), in spite of the fact that thorough molecular investigations on *T. hydatigena* have still to be carried out. The aim of the current study was to determine DNA sequence variation and generate a phylogenic tree for this helminth using the mitochondrial 12S rRNA gene sequence of *C. tenuicollis*, taking specimens from slaughtered sheep in the Sulaymaniyah province of Iraq as material. This investigation provided important data about strain dissemination in the study area and suggested that this could be comparable with that of strains in neighbouring countries, which may help to create a programme of understanding and control of *T. hydatigena* infection in the investigated area.

Material and Methods

***C. tenuicollis* isolates and DNA extraction.** A total of 46 samples of *C. tenuicollis* isolates were collected from sheep in 2017 during meat inspections at the Modern Sulaimani Slaughterhouse, Sulaymaniyah province, Iraq. Ethyl alcohol (70%) was used for specimen's disinfection and preservation. Genomic DNA was extracted from individual cysticerci using a PrimePrep Genomic DNA Extraction Kit (GeNet Bio Co., Daejeon, Korea) and stored at −20°C. DNA concentration was evaluated using a Genova Nano spectrophotometer (Jenway, Stone, U.K) and ranged between 20 and 55 ng/µL.

PCR amplification and purification of mitochondrial 12S rRNA. A set of primers, 12SRF (forward): 5 "-AGGGGATAGGACACAGTGCCAGC-3" and 12SRR (reverse): 5" -CGGTGTGTACATGAG CTAAAC-3" was used to amplify a fragment of the 12S rRNAgene. The DNA amplification was performed using *f-Pfu* DNA polymerase (SBS Genetech Co., Beijing, China) in a thermal cycler (Techne, Stone, U.K) under the conditions as described previously by Rostami *et al*. (18). The amplicons were assessed by electrophoresis in 1.5% (w/v) agarose gels using GoodView Nucleic Acid Stain (SBS Genetech Co.).

Among the PCR products, 19 concentrated DNA isolates were chosen for purification and partial sequencing using the forward primer. For further confirmation, double sequencing reactions were made for amplicons containing mutations using the reverse primer. Before sequencing, extracted DNA fragments from agarose gel were purified using SiMax PCR Products/Agarose Gel Purification Kit (SBS Genetech Co.). The purified DNA was commercially sequenced on an ABI 3730XL capillary machine (Applied Biosystems, Foster City, CA, USA). The Bioedit program (5) was applied to edit and align the acquired sequences, which were then submitted to the National Center for Biotechnology Information (NCBI) GenBank using Bankit (2). Their accession numbers (MK858233–MK858251) will be available in the GenBank database.

Phylogenetic analysis of 12S rRNA gene. Sequence analysis was undertaken by basic local alignment search tool (BLAST) algorithms and databases from NCBI (http://www.ncbi.nlm.nih.gov/). Genealogical relationships were determined by comparing all isolates obtained from the current study with other GenBank records for *T. hydatigena* and other taeniids (Table 1). The phylogeny of taeniid species was built by selecting the neighbour-joining (NJ) method available in the Molecular Evolutionary Genetics Analysis, version 7(MEGA 7) program (12). The Kimura two parameter model was implemented to compute the distance algorithm. Bootstrap interpretation was applied to examine the robustness of the tree topology, and the rates were evaluated with 1,000 replicates of the data sets.

Table 1. *T. hydatigena* and other Taeniid nucleotide sequences used for phylogeny

Species	Origin	Host	Accession number	Reference
T. hydatigena	Iran	Sheep	KU745527	Ibrahimi *et al.*, 2016 unpublished
T. hydatigena	Iran	Sheep	KX094339	Ibrahimi *et al.*, 2016 unpublished
T. hydatigena	Egypt	Sheep	KU671393	Abbas *et al.*, 2016 unpublished
T. hydatigena	China	Sheep	GQ228819	Jia *et al*., 2010 (9)
T. hydatigena	China	Dog	FJ518620	Liu *et al*., 2011 (14)
T. multiceps	Iran	Sheep	JQ710646	Rostami *et al*., 2013 (19)
T. ovis	Iran	Sheep	JX134130	Rostami *et al*., 2012 unpublished
T. saginata	Iran	Cattle	KC344681	Rostami *et al.*, 2012 unpublished
T. solium	China	Pig	AF337904	Li and Dianwu, 2001 unpublished
E. granulosus	Portugal	Sheep	HG975356	Beato *et al.*, 2014 unpublished

Results

All *C. tenuicollis* Iraqi isolates (n = 46) were successfully amplified for the 12S rRNA gene, and the amplified DNA fragment size was approximately 489 bp (Fig. 1).

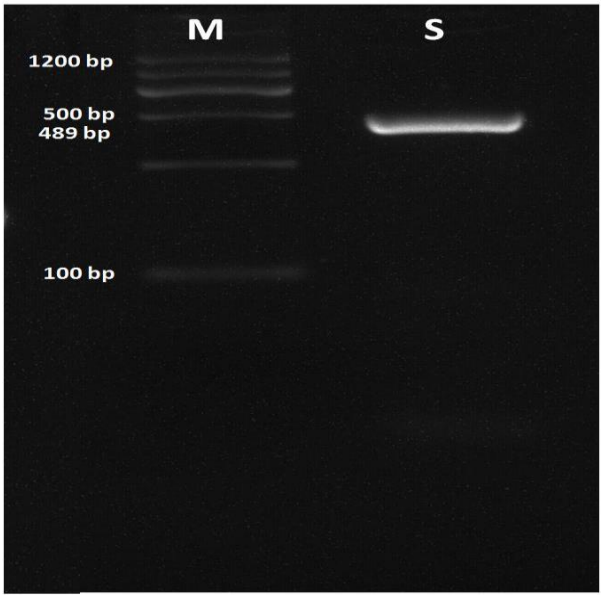

Fig. 1. PCR amplification of 12S rRNA gene. The12S rRNA gene (489 bp) of *C. tenuicollis* was amplified and resolved on 1.5% agarose gel. Lane M –marker (100-bp ladder DNA); lane S – positive *C. tenuicollis* isolate

Among the PCR products, 19 highly concentrated examples were randomly chosen and purified. The purified DNA samples were electrophoresed on 1.5% agarose gel to check the quality of the purification (Fig. 2). A total of 437 bp for the 12S rRNA nucleotide sequences of *C. tenuicollis* isolates (n = 19) were obtained. The DNA sequences were analysed, and the phylogenetic tree of 12S rRNA was constructed. The results of this study showed five haplotypes (H1–H5) of *T. hydatigena*, as represented in Fig. 3. For further analysis of haplotype diversity, the maximum composite likelihood method was used. The level of pairwise nucleotide variation showed the differences ranging from 0.2% to 0.7%, and the overall nucleotide difference was determined as 0.05% among the five haplotypes. Haplotype number 5 (H5) showed the highest nucleotide variation among *C. tenuicollis* isolates because it contained two base mutations (Table 2). Accordingly, phylogeny based on 12S rRNA showed that 14 out of 19 *T. hydatigena* isolates (accession numbers MK858233–MK858246) were 100% identical to a *T. hydatigena* species isolated from Iranian sheep recorded under accession number KU745527, while the remaining isolates were slightly different and showed 99.54% to 99.77% identity to it (Fig. 4).

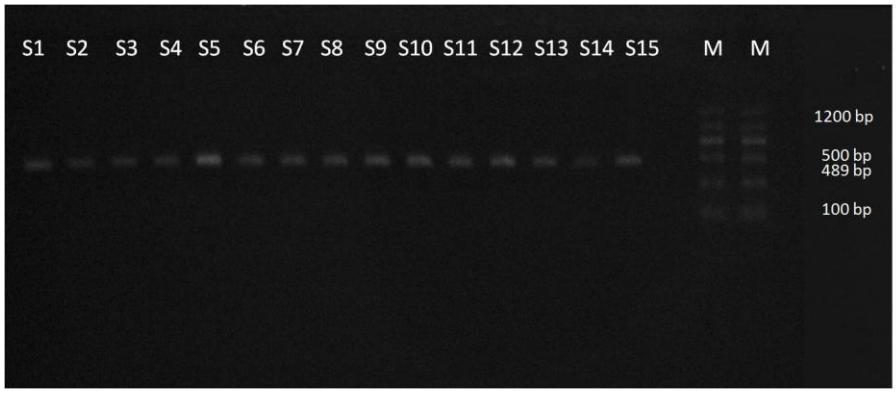

Fig. 2. DNA purification of 12S rRNA gene. PCR products of 12S rRNA gene were recovered and electrophoresed on 1.5% agarose gel. Lane M – marker (100-bp ladder DNA); lanes S1-S15 – positive *C. tenuicollis* isolates

Table 2. Pairwise nucleotide variations among the five haplotypes of the 12S rRNA gene (determined using the maximum composite likelihood method)

	H5	H4	H3	H2	H1
H5[e]					
H4[d]	0.007				
H3[c]	0.007	0.005			
H2[b]	0.007	0.005	0.005		
H1[a]	0.005	0.002	0.002	0.002	

H1[a] – haplotype 1; H2[b]– haplotype 2; H3[c]– haplotype 3; H4[d]– haplotype 4; H5[e] – haplotype 5

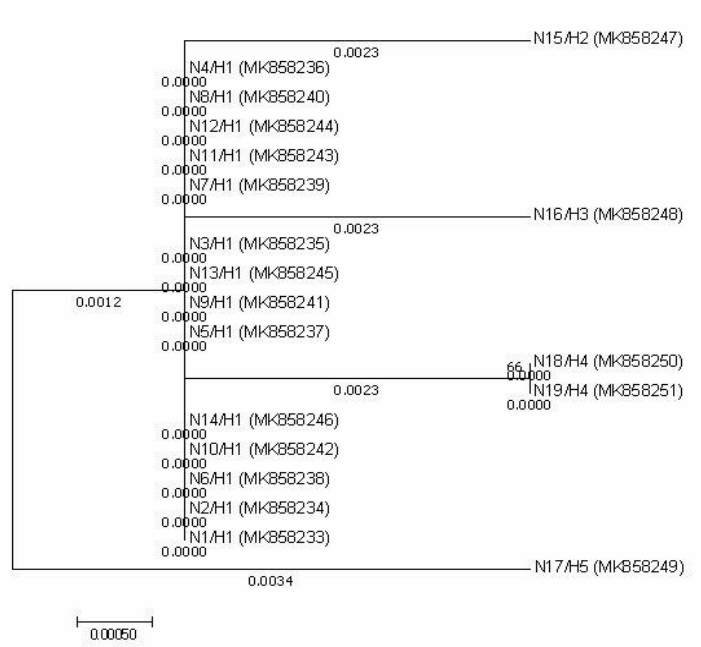

Fig. 3. Intraspecific phylogenetic relationship of sheep *C. tenuicollis* isolates in Iraq. The phylogeny of sheep *C. tenuicollis* was computed by neighbour joining (NJ) from the partial 12S rRNA gene nucleotide sequences. The scale bar represents 0.05% divergence. Bootstrap values are shown above or below branches. MK858233–MK858251 are GenBank accession numbers representing *T. hydatigena* sequences identified in this study; N1–N19 isolated *C. tenuicollis*; H1–H5 haplotypes of *C. tenuicollis*

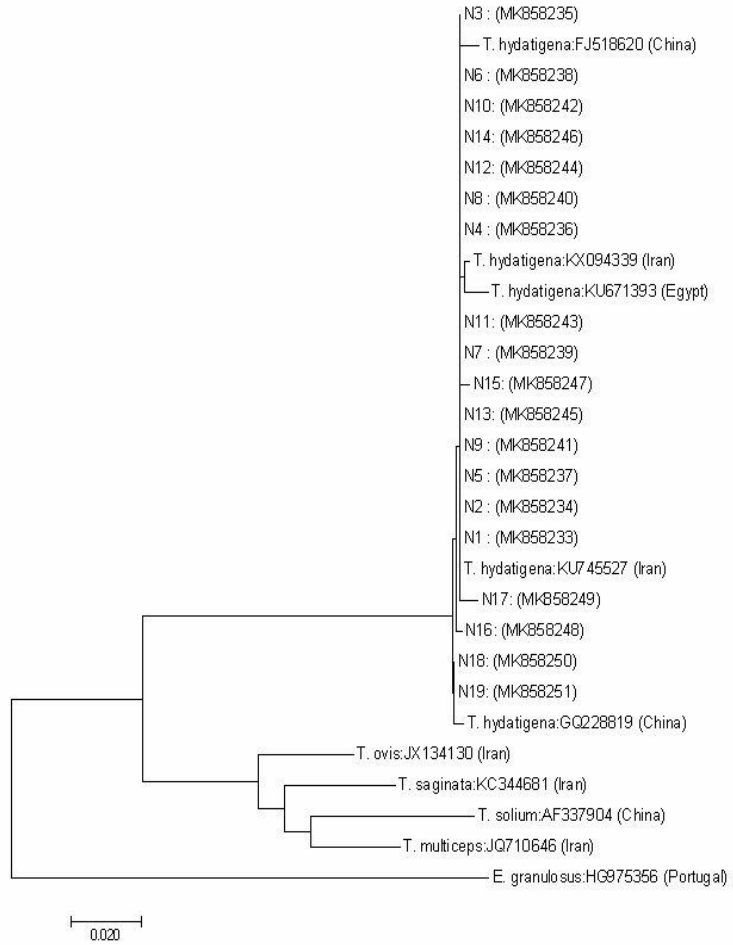

Fig. 4. Interspecific phylogenetic relationship of *C. tenuicollis*. Phylogeny of *T. hydatigena* isolates represents partial 12S rRNA sequences. The scale bar represents the estimated numbers of nucleotide substitutions per nucleotide site

Discussion

To the best of our knowledge, this is the first study on the genetic diversity and population structure of *C. tenuicollis* using 12S rRNA. By sequence analysis of this rRNA gene, all isolated cysts were found to be *T. hydatigena*, confirming the macroscopic diagnosis (20). A few past investigations based on morphological and biochemical elements gained more exact knowledge of the genetic diversity inside the *Taenia* species (3, 7, 15). The results of the current study indicated relatively minor nucleotide differences in 12S rRNA among *T. hydatigena* isolates of the study region. Pairwise nucleotide variation in the 12S rRNA gene was 0.2%–0.7% and was not as wide as the 0.2%–2.1% recorded among Iranian isolates (18). The polymorphism in haplotype diversity seen for this area may be linked to the high prevalence helminths, epidemiology, and dispersal of the host along with husbandry systems. The low intra- and interspecific differences seen in the present study suggest the absence of major genetic differences among *C. tenuicollis* isolated from sheep in Sulaymaniyah, Iraq.

In this study, mitochondrial DNA (mt-DNA) sequence data was used to examine the intra-specific variation of *T. hydatigena*. Mt-DNA is widely used in molecular and phylogenetic studies of eukaryotic organisms due to its low or absent recombination, maternal inheritance, conserved structure, absent in existence of introns, greater mutation, and adequate evolutionary rate (1, 16). The most common mtDNA gene for analysing the phylogeny, inter- and intra-specific divergence and evolution of parasitic helminths is 12S rRNA (3, 21). According to the results obtained during the current study, four new strains of *C. tenuicollis* containing one or two base mutations were discovered in Iraq for the first time. Three strains contained only a single mutation and the other one contained two. In addition, these mutations were not found in NCBI Blast searches, so they may be a unique mutations globally which has not been recorded previously. These new mutations manifested differences from strains recorded in other countries (9, 18), but the strains showed the highest similarity, ranging from 99.54% to 99.77%, with a strain isolated in Iran (accession no. KU745527). This outcome is not surprising as the region shares a border with Iran. The genetic diversity of the strains is very low compared to that of the Iranian strains.

Phylogenetic study of the 12S rRNA gene created a tree with similar topology for *T. hydatigena* isolates; however, the general topology of the 12S rRNA tree is different for other *Taenia* species. In the 12S rRNA phylogenetic tree, all the *T. hydatigena* isolates recovered in this study were clustered in one clade, along with isolates from Iran, China, and Egypt. The tapeworm *T. hydatigena* is one of the universal taeniid species among ruminants and canines, and the helminth seems to have been globally distributed through human colonisation and livestock movement over an extensive duration. Intensive transmission of the parasite among a range of intermediate host species could enhance the opportunity for genetic discrepancy within different populations of the parasite in nature.

The present investigation established new data for mt-DNA sheep isolates of *T. hydatigena* in Iraq. The phylogenetic analysis of 12S rRNA gene computed by neighbour joining revealed that *C. tenuicollis* was composed of five haplotypes clustered in one clade. Four new strains containing new mutations were found in the study area. More in-depth studies on nuclear genes are essential to provide a comprehensive picture on the extent and significance of genetic variation within different *T. hydatigena* populations.

Conflict of Interests Statement: The author declares that there is no conflict of interests regarding the publication of this article.

Financial Disclosure Statement: This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Animal Rights Statement: None required.

Acknowledgements: The author would like to thank Dr. Taib Ahmad Hama-Soor in Sulaimani Polytechnic University for his continuous assistance in solving technical problems during this work and the critical revision of this manuscript.

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