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Original Article

Novel isolate of *Cysticercus tenuicollis*-Kalar isolate has been revealed in Kalar, Iraq

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Abstract

Introduction: Although *Cysticercus tenuicollis* is one of the most economic and veterinary important parasite in Iraq, scanty molecular characterization exists for this helminth. This study aimed to determine the prevalence and molecular description of *C. tenuicollis* isolates from sheep in Kalar district of Iraq.

Methodology: A total of 2,906 slaughtered sheep were examined post-mortem. Up to 20 samples of *C. tenuicollis* was extracted and amplified using mitochondrial COX1 gene.

Results: The overall prevalence rate was 6.88%, and female sheep recorded higher rate of infection (24.35%) than male (6.16%) with significant difference ($p<0.05$). The molecular results showed 14 haplotypes for COX1 gene and the pairwise nucleotide variation among them was ranged from 0.2 to 2.6%. Twelve out of fourteen haplotypes of *C. tenuicollis* involving one to three base mutations were discovered in Kalar, Iraq for the first time and this could be a unique mutation internationally and did not registered previously. Eleven newly recorded haplotypes involved only one single mutation and the remaining one involved three mutations. Phylogenetic interpretation showed that *Cysticercus tenuicollis*-Kalar isolate were clustered in one clade, and closely related to isolates discovered in Nigeria, China, Turkey, Poland, and Iran.

Conclusions: This study provided a new record data on prevalence and discovered novel strains of *C. tenuicollis* in the study area for the first time named *Cysticercus tenuicollis*-Kalar isolate. Novel haplotypes might consider endemic genetic characterization of this metacestode. The present data may be useful to provide a good molecular background for future preventive and control programs.

Key words: Prevalence rate; haplotype; genetic variation; phylogenetic tree.

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Introduction

Abdominal cysticercosis is caused by *Cysticercus tenuicollis*, the metacestode stage of *Taenia hydatigena,* in a wide variety of intermediate hosts, for example domestic and wild ruminants [1,2]. The adult parasite of this metacestode is discovered in the intestine of domestic and wild carnivores.Ruminant intermediate hosts get the infestation by eating ova through contaminated food and water. Following the ova hatch in the digestive tracts, oncospheres are discharged and shipped to the visceral cavity by means of blood flow [3,4]. Immediately after migrating through the liver of the intermediate host, the metacestode of *T. hydatigena*, typically encyst on the omentum, mesentery and the serosal surface of the liver; however, they can also be found in the lungs, heart, uterus or kidneys [1]. Subsequent migration of the larvae can result in traumatic hepatitis, resulting in the death of the host animal [3]. Radfar *et al.* [5] observed *T. hydatigena* metacestodes in the thoracic cavity only in 1.24% of examined animals while most of data refer to larvae of *T. hydatigena* in the abdomen cavity [1,4,6]. Cysticercosis by *T. hydatigena* results in serious economic impacts on livestock production due to condemnation of animal offal's or whole carcass at slaughter, which makes it a problem of veterinary significance [6]. The diagnosis of visceral cysticercosis is depended on morphological and molecular characterization of *T. hydatigena* metacestodes, for instance, blade length, hook number and hook size. Today, molecular assay are generally utilized to segregate between various species of parasites, and there are a number of investigation focused on cystic stage of *T. hydatigena* in domestic ruminants such as sheep, goat and cattle [2,3,7-9].

The aim of current work was to determine the frequency rate and molecular description (strain identification and phylogenic analysis) of *C. tenuicollis* using mitochondrial COX1 gene among sheep at Kalar slaughterhouse of Kalar district, Iraq.

Methodology

Study area and sample collection

This study was done for the first time in 2020 in Kalar slaughterhouse belonging to the Kalar district in Garmian area. It is located in the southeast Kurdistan region of Iraq and home to around 120 thousand sheep that are managed majorly by traditional method of livestock farming. A total of 2,906 sheep were inspected between January and March 2020 by daily visiting slaughterhouse. The randomly examined sheep were 2,791 males and 115 females. In the study area, the majorities of slaughtered sheep were males becausefemale sheep will pay attention for breeding and only slaughtered when reached an old age. Therefore, it is difficult to be accurate in sampling method considering almost similar number of males and females. The visceral organs were inspected for *C. tenuicollis* and identified according to morphological features [3]. Twenty samples of *C. tenuicollis* isolates from sheep were collected and preserved in 70% Ethyl alcohol till DNA extraction.

DNA extraction and PCR amplification of mitochondrial COX1 gene

A total of 20 cysts were extracted using *EasyPure*™ Genomic DNA Kit (Trnas Gen Biotech Co., Beijing, China). The extracted DNAs were measured by using Genova Nano Spectrophotometer (Jenway, U.K) and the concentration of the samples ranged from 15- 60 ng/µl.

A pair of primer, THCOIF (forward): 5'-TTGATCCATTAGGTGGTGGAG-3'and THCOIR (reverse):5'TCCAGTAATTAAAGGTCACCATC-3' were used to amplify mitochondrial COX1 gene. The DNA amplification was performed by using *f-Pfu* DNA Polymerase (SBS Genetech Co., Beijing, China) under the conditions in the thermal cycler (Techne, U.K) as previously described by [10]. The amplicons were verified by gel electrophoresis on 1.5% agarose gels (TBA, 0.5%) stained with GoodView™ Nucleic Acid Stain (SBS Genetech Co., Beijing, China).

DNA purification and partial sequencing of COX1 gene

All PCR products (20 amplicons) were subjected to purification and sequencing using upstream primer. For additional affirmation, double partial sequencing reactions were done for products involving mutations using downstream primer. Purification of extracted DNA fragments was performed from agarose gel by using SiMax™ PCR Products/ Agarose Gel Purification Kit (SBS Genetech Co., Beijing, China). All purified DNA were partially sequenced by Sanger sequencing method (CHU de Québec-Université Laval, Québec city, Canada). Sequences were edited and aligned using Clustal W multiple sequence alignments as applied in BioEdit software [11], then deposited it in National Center for Biotechnology Information (NCBI) and available in Genbank database under accession numbers MT086484-MT086503.

Molecular analysis of COX1 gene

All nucleotide sequences were blasted by basic local alignment search tool (BLAST) algorithms and analyzed with databases from NCBI (http://www.ncbi.nlm.nih.gov/). Phylogenetic tree of COX1 gene was constructed by comparing all isolates obtained from the present study with other *T. hydatigena* GenBank accession numbers retried from NCBI database (Table 1). The phylogeny of Iraqi isolates was built by selecting the neighbor-joining (NJ) method available in Molecular Evolutionary Genetics Analysis version 7 (MEGA7). Kimura 2-parameters

Table 1. The nucleotide sequences of *T. hydatigena* from Genbank used for phylogenetic comparison in this study.

Accession number	Origin	Host	Reference
MN175592 MN175593 MN175594 MN175595 MN175596	Nigeria	Sheep and Goat	Ohiolei*et al.,* 2019 [13]
JN831291 JN831294 JN831295 JN831297 JN831298 JN831304	China	Goat	Hao *et al.,* 2011 unpublished
JN831309		Pig	
MF630923 MF630926	Poland	Wild Boar	Filip *et al.,* 2019 [14]
MK851045	Turkey	Red Deer	Cengiz *et al.,* 2019 [15]
KR337823	Iran	Goat	Karamian *et al.,* 2015 unpublished

model was implemented to compute the genetic distances. Bootstrap interpretation exerted to examine the robustness of the tree topology. The rates of bootstrap were evaluated with 1000 replicates of the data sets [12]. For additional analysis, nucleotide and haplotype diversity were calculated by Maximum Composite Likelihood model.

Statistical interpretation

The prevalence data analyzed by Chi-square (χ^2) test using MS Excel (2010). Values of $p < 0.05$ were considered statistically significant.

Animal rights statement

The authors declare that the experiments on animals were conducted in accordance with local Ethical Committee laws and regulations as regards care and use of laboratory animals.

Results

The overall infestation rate of slaughtered sheep by *C. tenuicollis* in Kalar slaughterhouse was 6.88% (200/2906). Based on sex, there was significant difference ($p < 0.05$) between male 6.16% (172/2791) and female 24.35% (28/115) as shown in Table 2. The difference between infestation rates of organs and sexes was shown in Table 3, statistically no significant difference ($p > 0.0$) was observed between infestation rate of liver (3.88%) and mesentery (3.00%).

The DNA of twenty isolates (N=20) of *C. tenuicollis* were successfully extracted and PCR was used to amplify mitochondrial COX1 gene (551 bp) (Figure 1). The amplicons were gel recovered and sequenced through Sanger sequencing. Following editing and trimming of derived sequences, a total of 507 bp for the COX1 nucleotide sequences of *C. tenuicollis* isolates (N=20) were obtained. The DNA nucleotide sequences were analysed and phylogeny of COX1 gene was constructed. The results of the present work found 14 haplotypes (H1-H14) of *T. hydatigena* as illustrated in Figure 2. For additional analysis of haplotype diversity, the Maximum Composite Likelihood model was used. The level of pairwise nucleotide variation showed the differences ranging from 0.2 to 2.6% and the overall distance was 0.9% among haplotypes. Haplotype number 14 (H14) showed the highest nucleotide variation among *C.*

Figure 1. PCR amplicon of mitochondrial COX1 gene (551 bp) of *C. tenuicollis* was amplified and run on 1.5% agrose gel. Lane L:ladder (100-bp ladder DNA); Lane +S:positive *C. tenuicollis* (*Taenia haydatigena*) isolate.

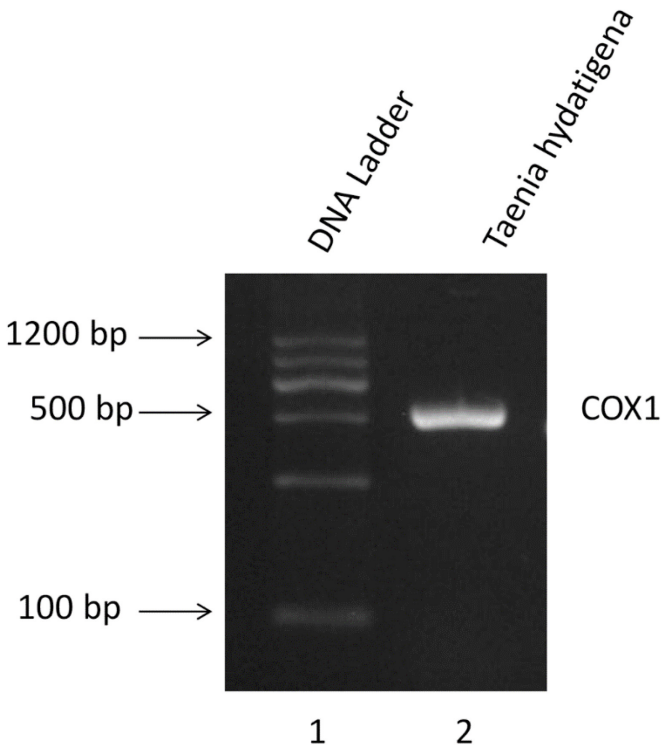


Table 2. Infestation rates of *C. tenuicollis* among slaughtered sheep (N[a] = 2906) in Kalar slaughterhouse, Iraq

Total number	No. examined	No.(%) infected	χ^2 (P-value)
	2906	200 (6.88)	
Sex			
Male	2791	172 (6.16)	p <0.001
Female	115	28 (24.35)	

[a]N: total number of examined sheep.

Table 3. The variation between infestation rates of *C. tenuicollis* of specific organs and sex.

Organs	No. examined	No. (%) positive	χ^2 (P-value)	Organs	Sex	No. examined	No. (%) positive	χ^2 (P-value)
Liver	2906	113 (3.88)	p = 0.06	Liver	Male	2791	97 (3.47)	p <0.001
					Female	115	16 (13.91)	
Mesentery	2906	87 (3.00)		Mesentery	Male	2791	75 (2.69)	
					Female	115	12 (10.44)	

Table 4. Pairwise nucleotide divergence between haplotypes of *C. tenuicollis.*

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
H1														
H2	0.002													
H3	0.002	0.004												
H4	0.002	0.004	0.004											
H5	0.002	0.004	0.004	0.004										
H6	0.002	0.004	0.004	0.004	0.004									
H7	0.002	0.004	0.004	0.004	0.004	0.004								
H8	0.002	0.004	0.004	0.004	0.004	0.004	0.004							
H9	0.002	0.004	0.004	0.004	0.004	0.004	0.004	0.004						
H10	0.002	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004					
H11	0.002	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.004				
H12	0.018	0.020	0.020	0.020	0.020	0.020	0.020	0.020	0.016	0.016	0.020			
H13	0.018	0.020	0.020	0.020	0.020	0.020	0.020	0.020	0.016	0.016	0.020	0.004		
H14	0.008	0.006	0.010	0.010	0.010	0.010	0.010	0.010	0.010	0.010	0.010	0.026	0.026	

H1-H14: Haplotype 1 to haplotype 14; The overall mean distance: 0.009.

tenucollis haplotypes because it was containing three base mutations (Table 4). Accordingly, phylogeny based on COX1 gene showed that 8 out of 20 *T. hydatigena* isolates of this study were 100% identical to *T. hydatigena* strains isolated from countries including Nigeria, Turkey, Poland and China, whereas the other isolates were somewhat variant and revealed 99.41 to 99.80% identical to strains isolated from Nigeria, Turkey, Poland, China, and Iran (Figure 3).

Figure 2. Phylogenetic tree of sheep *C. tenuicollis* isolates in Kalar-Iraq calculated by Neighbor- joining (NJ) method from the partial mitochondrial COX1 gene sequences. The distance bar shows 0.2% divergence of the sequences. MT086484-MT086503 GenBank accession numbers represent *C. tenuicollis* sequences identified in present study; N1-N20: isolated *C. tenuicollis*; H1-H14: haplotypes of *C. tenuicollis.*

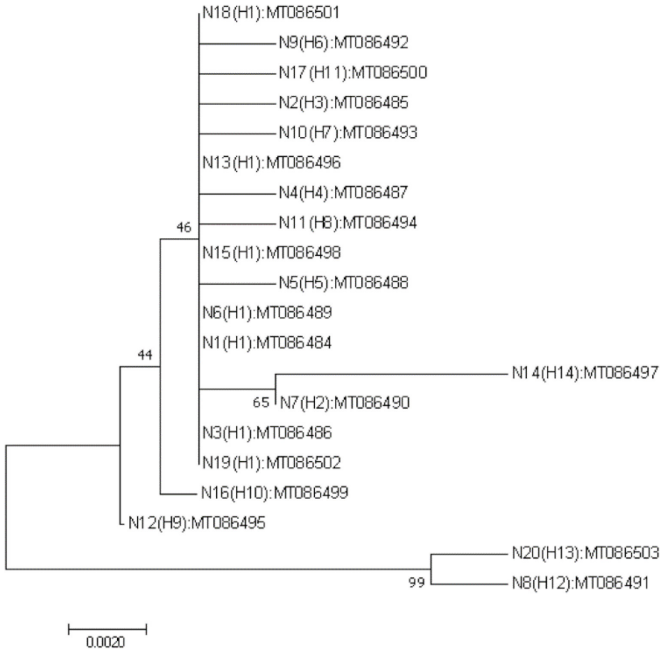
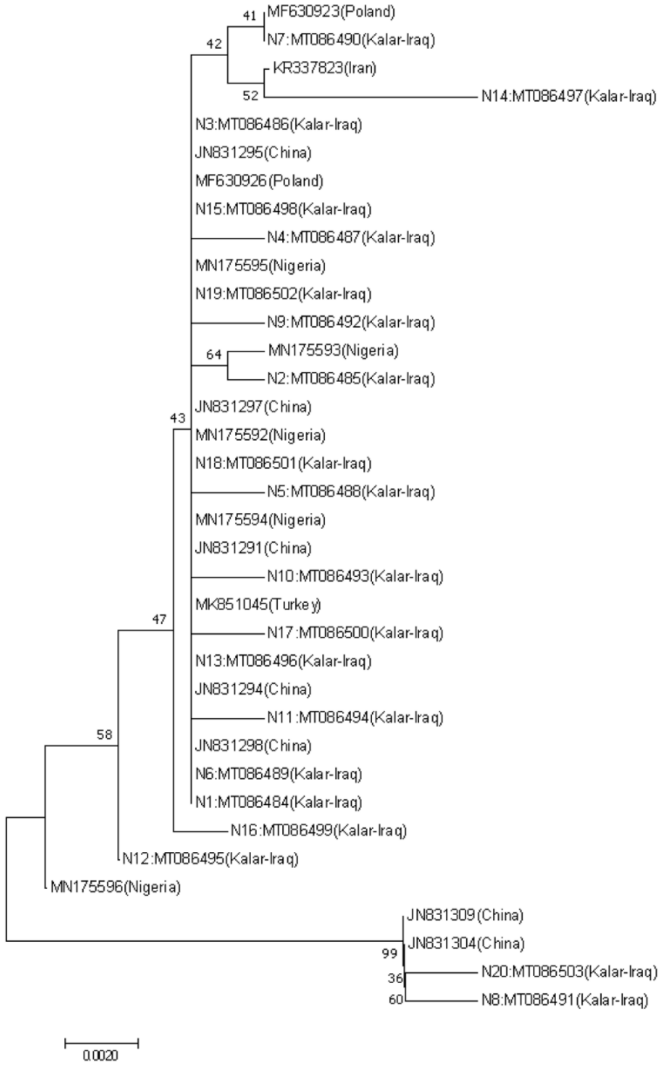


Figure 3. Phylogenic relationship of sheep *C. tenuicollis* isolates in Kalar-Iraq with other identical isolates in the world. Phylogeny of *C. tenuicollis* isolates shows partial mitochondrial COX1 sequences. The scale bar represents 0.2% difference between nucleotide sequences.



Discussion

In this study, the overall rate of *C. tenuicollis* infection in sheep slaughtered at Kalar slaughterhouse, Iraq was 6.88%, this result is considered a lower frequency rate compared to other published studies that conducted in Iraq; the infection rates were 40.55% in Basrah [16], 32.50% in Karbala [17], 22.60% in Sulaymaniyah [18], and 14.22% in Baghdad [19]. The authors interpreted the reasons of the high prevalence rate of this parasite among sheep could be due to inadequate knowledge and awareness about this disease among the Iraqi people and a shortage of medical incinerator in the most slaughterhouses to collect and burn the infected animal offal's or carcasses. Also illegal slaughtering of animals, improper disposable of infected organs and huge number of stray dogs make difficult to control this parasite. On the other hand, the result of current study was higher than recorded by each of [20] in Duhok (0.7%) and [21] in Mosul (2%). However, the result of [22] recorded in Al-Qadisiya (7.4%) which supports the present study. This variation could be due to degree of pasture contamination, animal management, feeding habits, grazing patterns, and environmental factors. Also the knowledge about the disease, separating dogs from livestock, and regular cleaning of farm animal had crucial role on reducing the infection.

It is possible that hunting dogs fed with offal from ungulates or stray dogs fed with carrion may remain at serious risk of infection [23]. All infected animals came from rural areas, which can be evidence that dogs, on contact with wild animals and carrion, should be considered as an important source of *T. hydatigena* infection. Also jackals, which have begun to expand in Poland recently may play a role in spreading *T. hydatigena* infection [24]. Therefore, it is essential to begin regular monitoring of *T. hydatigena* infection in both wild and domestic animals in Iraq and to determine which canids are involved in spreading the cestode in the wild.

The frequency of infection by sex showed that higher infestation was found in female (24.35%) compared to male (6.16%) with significant difference, this is agreed with the study recorded by [16]. It may be due to the consequence of longer life cycle of female ewes for breeding and most of the females are slaughter older. In addition to that, sampling method was unbalanced in which higher number of males (2791) randomly examined compared to females (115). Based on distribution of cyst, the infection rate of *C. tenuicollis* was higher in liver (3.88%) than mesentery (3.00%), this finding is similar to the observation found by Essa *et al.* [16]. This may be due to the presence of large amount of protein, carbohydrates and other essential elements which absorbed by the parasite, so that, *C. tenuicollis* prefer liver as organ of supplying essential elements for nourishment.

To the best knowledge, this is the first report on molecular characterization and phylogenetic tree of *C tenuicollis* in the study region using mitochondrial COX1 gene. By amplification of PCR products and sequence analysis of COX1 gene, all isolated samples were found to be *T. hydatigena* metacestodes, affirming the macroscopic diagnosis [25]. Previously, morphological and biochemical parameters investigation of the parasite fortified the presence of genetic diversity within *Taenia* species [26,27]. The results of this study showed a low level of nucleotide differences of COX1 among *T. hydatigena* isolates, the Pairwise nucleotide variation of COX1 gene was arranged from 0.2–2.6% and this difference was not very wide as recorded among isolates of Iranian sheep, 0.3-3.4% [3], this polymorphism seen in haplotype diversity in Iranian sheep could be related to the high infestation rate of the parasite, epidemiology and dispersal of host along with husbandry system. The low intra-specific and inter-specific variation found in the currentstudy proposes a low genetic difference among *C. tenuicollis* isolated from sheep in Kalar district of Iraq.

Mitochondrial DNA (Mt-DNA) sequence data was used in this study to inspect the intra-specific diversity of *T. hydatigena* isolates. Mt-DNA is broadly used in molecular and phylogenetic analysis of living eukaryotic due to its low or inexistence recombination, maternal inheritance, conserved structure, miss of introns, high mutation and great rate of evolution [28]. The most popular mt-DNA genes are COX1 and 12S rRNA genes for phylogeny, inter-specific and intra-specific differences and evolution in biology of parasitic helminthes [27,29]. Referring to the finding acquired during the present work, 12 novel strains (H3 – H14) of *C. tenucollis* involving one to three base mutations were revealed in Kalar district of Iraq for the first time. 11 out of 12 strains involved only one single mutation and the remaining one contained three mutations. Furthermore, these new mutations were not found in NCBI Blast browser, so it might be the novel mutation internationally and did not recorded already. This single mutation was repeated in 11 cysts' isolated from 11 different sheep and this result validates the precise result. Therefore, this new isolate was named *Cysticercus tenuicollis*-Kalar isolate which is endemic and specific to the study area. These novel mutations

made a variation with strains reported in other countries [13-15],although it showed the highest similarity ranged from 99.41 to 99.80% with strains isolated from Iran (GenBank accession no: KR337823), Poland (GenBank accession no: MF630923), Nigeria (GenBank accession nos: MN175592-MN175596), Turkey (GenBank accession no:MK851045), Poland (GenBank accession no:MF630926), and China (GenBank accession nos:JN831291-JN831292, JN831294-JN831295, JN831297-JN831298). In phylogenetic tree of the COX1 gene, all the Kalar isolates of *T. hydatigena* recovered in this study were clustered in one clade, along with isolates from Nigeria, Turkey, Poland, China, and Iran. This result confirms the circulation of *C. tenuicollis* from different geographical regions.

Conclusions

The present study concludes that the infection exists in the study area and it is endemic. The novel isolates were discovered in the area, *Cysticercus tenuicollis*-Kalar isolate, after detecting a single mutation in a conservative mitochondrial COX1 gene of 11 isolates. The findings of the present study are important to understand the epidemiology and to conduct future strategy to control recognized parasite in kalar district of Iraq.

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