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New insights into the prevalence and phylogenetic diversity of *Cysticercus ovis* isolates in sheep from Sulaymaniyah, Iraq

Aram Ahmad Mohammed

Microbiology Department, College of Veterinary Medicine,
Sulaimani University, Sulaymaniyah Province, Iraq
aram.mohammed@univsul.edu.iq

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Abstract

Introduction: Although ovine cysticercosis is not a zoonotic problem, it results in substantial economic losses due to the condemnation of infected tissues or entire carcasses. This study aimed to record preliminary data on the prevalence, and phylogenetic diversity of *Cysticercus ovis* isolates from slaughtered sheep in the province of Sulaymaniyah, Iraq. **Material and Methods:** From January to September 2020, 6, 411 slaughtered sheep were examined for *C. ovis* by routine meat inspection. The amplification and sequence analysis of the COX1 gene for up to 35 specimens of *C. ovis* was performed using conventional PCR. **Results:** The overall prevalence rate was 1.3%, and the prevalence was significantly higher in older sheep (>1 year) than younger ones (<1 year) ($P< 0.05$). The cardiac muscle showed a higher tendency to carry *C. ovis* infection compared to other examined muscles. Sequence analysis of the COX1 gene revealed six haplotypes, and the level of pairwise nucleotide diversity between individual haplotypes was 1–2%. Five out of six of the *Taenia ovis* haplotypes recovered could have been recorded for the first time globally. Phylogenetic interpretation indicated that all the *T. ovis* haplotypes clustered in a single clade, and it also indicated an extremely close similarity to Iranian and New Zealand isolates. **Conclusion:** Globally, this report adds new data on *C. ovis* genetic diversity, which provide an extremely useful molecular background with regard to future preventive as well as control strategies.

Keywords: prevalence, phylogenetic diversity, *Cysticercus ovis*, sheep, Iraq.

Introduction

The tapeworm *Taenia ovis,* which belongs to the family Taeniidae, is transmitted between carnivores as the definitive host and small ruminants as the intermediate host. When present in the heart and skeletal muscles of infected goats or sheep, the metacestodes of *T. ovis* cause considerable economic losses due to condemnation of carcasses infected with this parasite, also known as sheep measles (5, 6). Small ruminants become infected by grazing on pasture contaminated with the embryonated eggs that have been deposited in canine faeces. *Cysticercus ovis* occurs as a thin-walled, fluid-filled cyst approximately 1 cm in diameter inside the heart and skeletal muscles of the intermediate host. The cyst in the muscle degenerates and calcifies over time, forming a tiny nodule. Domestic and wild carnivores as the definitive hosts are infected through ingestion of ovine muscle tissue containing viable cysts (4).

Although ovine cysticercosis is neither a flock nor a zoonotic issue, it affects the quality of food. Both calcified and viable cysts are unpleasant to eat and can contribute to the downgrading or even condemnation of carcasses at the abattoir (4). As a consequence of detected cysticercosis, carcasses are condemned at slaughter following the Food and Agricultural Organization (FAO) guidelines (13). These suggest that a carcass be condemned because of infection with *C. ovis* if lesions are observed at two of the normal sites of inspection (tongue, masseter muscle, oesophagus, heart, diaphragm, or exposed musculature) and at two sites on making incisions in the shoulder and rounds (13). Thus, infection with *C. ovis* is a major concern and obstacle to profitability in the sheep industry, particularly in endemic regions (15).

In Iraq, no genetic information is available regarding *T. ovis*, while globally, there is also not much data related to the genomic structure of this parasite (24). More thorough molecular research in the field is required in order to provide new and more efficient vaccines against those parasites as well as improve our understanding regarding such species' genetic diversity. Mitochondrial genes, which are haploid (clonal), are maternally inherited. Mitochondrial DNA within individual organisms is considered to be subject to higher rates of mutation and genetic variability. In addition, it has been established that investigative procedures using mtDNA were vital to examine intra- and inter-specific variations in the parasitic species (8, 29).

The advancements in molecular genetic methods have resulted in the development of sophisticated tools to identify and examine the relations between taeniid species. In particular, mitochondrial DNA sequencing was effectively utilised for the genetic characterisation and identification of these parasites (3).

Infection with *T. ovis* occurs globally, and research has documented it in Canada (5), China (25), England (6), Ethiopia (26), Iran (12, 23), Egypt (1), and New Zealand (15). In Iraq, the epidemiology of *C. ovis* remains unknown, and there was no report describing this parasite in sheep. Therefore, this research aimed to record preliminary information on the phylogenetic diversity and prevalence of *C. ovis* isolates in slaughtered sheep from Sulaymaniyah province, Iraq.

Material and Methods

Field study area. The study was conducted from January to September 2020 in the Modern Sulaimani abattoir in the province of Sulaymaniyah, Kurdistan, in the northeast part of Iraq. The province is located between longitudes 44°50′ and 46°16′ and latitudes 35°04′ and 36°30′ and has an average of 400 m above sea level. The climate of the study area can be typified by seasonal rainfall between October and May. The sheep population is about 1.2 million based on data Sulaymaniyah Veterinary Directorate data from December 2015.

Study animals and specimen collection. A total of 6,411 sheep were randomly examined in this study during regular visits to the abattoir twice per week. The choice of sample size was based on a confidence interval (CI) of 95% and a margin of error of 5%. Before slaughter, the sex and age of each sheep was recorded (<1 year designated young or >1 year designated adult, based on dentition). The carcass of each animal was subjected to a standard meat inspection procedure after slaughter. For *C. ovis* cyst presence, the heart muscle, diaphragm, intercostal muscles, and abdominal muscles were examined *via* visual examination, palpation and incisions. Morphology, specifically the size and shape of the cyst and the scolex, including the rostellar hooks, was the basis for the identification of *C. ovis* (5). For further processing, the muscles which contained *C. ovis* cysts were placed in sterile containers with 70% ethyl alcohol and transferred to the laboratory of the Research Center, at the College of Veterinary Medicine, Sulaimani University.

DNA extraction and PCR amplification of mitochondrial COX1 gene. DNA from 35 *C. ovis* cysts was extracted using an *EasyPure*™ Genomic DNA kit (Trans Gen Biotech Co., Beijing, China) following the manufacturer's instructions. The extracted DNA isolates were measured using a Genova Nano Spectrophotometer (Jenway, Stone, U.K), and the concentration of the samples ranged from 15 to 55 ng/µL.

An approximately 400 bp-long fragment of the mitochondrial COX1 gene was amplified by means of a primer pair, comprising JB3 5′-TTTTTTGGGCAT CCTGAGGTTTAT-3′ as the forward one and JB4.5 5′-TAAAGAAAGAACATAAATGAAAAATG-3′ as the reverse one (2). The mitochondrial DNA was amplified using *f-Pfu* DNA polymerase according to the manufacturer's protocol (SBS Genetech Co., Beijing, China), under the conditions previously described (23). The PCR products were confirmed *via* gel electrophoresis on 1.5% agarose gels (TBA, 0.5%) that were stained with GoodView Nucleic Acid Stain (SBS Genetech Co.).

PCR product purification and sequencing of the COX1 gene. The 35 amplicons were subjected to purification and sequencing with the use of the forward primer. For additional validation, double-partial sequencing reactions were conducted for products presenting mutations utilising the reverse primer. In addition, the purification of extracted DNA fragments was performed from the agarose gel utilising SiMax PCR Products/ Agarose Gel Purification kit (SBS Genetech Co.). Furthermore, all the purified DNA samples were partially sequenced with the Sanger method, using anABI-3700 DNA Analyzer (Applied Biosystems, Foster City, CA, USA). Sequences were edited and aligned using ClustalW multiple sequence alignments in BioEdit software (10). The nucleotide sequences of the COX1 gene were submitted to the NCBI and are available in the GenBank database under accession numbers MW017206–MW017211.

Phylogenic analysis of the COX1 gene. The obtained nucleotide sequences of *T. ovis* were found in the basic local alignment search tool (BLAST) algorithms and analysed with NCBI databases. A phylogenic tree was constructed on the basis of comparison and alignment with the reference sequences of other Taeniidae organisms with the use of the neighbour-joining approach of coding genes in the *T. ovis* mitochondrial genome (Table 1). The genetic distances were calculated utilising Kimura's two parameter model and the robustness of the tree topology was measured by using the bootstrap value of 1,000 replicates of the data sets available in version 7 of MEGA 7 (14). For additional analysis, the pairwise diversity of nucleotide sequences and haplotypes was computed by the maximum composite likelihood model (14).

Data interpretation. The data were analysed by X^2 test and confidence intervals (CI) with the use of SPSS software version 25 (IBM Corporation, Armonk, NY, USA). Probability values below 0.05 were deemed statistically important.

Results

Among 6,411 sheep inspected, 84 (1.31%; 95% CI 0.21–2.41%) were infected with *C. ovis* cysts. In terms of sex, the prevalence in male sheep (1.57%) was not significantly higher than in female sheep (1.00%). Based on age, infection in adult sheep over one year old (1.73%) was significantly (P< 0.05) higher than in young sheep under one year old (0.83%), as displayed in Table 2. The dispersal by infection site, shows the cardiac muscle having the maximum rate of infection (1.26%) (Table 3).

A PCR product of 382 bp was obtained following the editing and trimming of the studied sequences. Sequence analysis and phylogeny of *T. ovis* showed that the overall number of mutations was 11 in the COX1 gene and that they existed at 11 polymorphic sites. The COX1 gene of this study showed six haplotypes assigned as IQTOH1–IQTOH6 (Table 4). The pairwise nucleotide difference between COX1 individual haplotypes was 1–2%, whereas the total nucleotide difference between the six haplotypes was 1% (Fig. 1). The phylogenic tree based on COX1 sequence data analysis revealed that all *T. ovis* isolates were located in a single clade of the tree (Fig. 2).

Table 1. The nucleotide sequences of *T. ovis* and other taeniids from GenBank used for phylogeny

Species of parasite	Accession No.	Country	Host	Citation (reference)
T. ovis	AB731675	New Zealand	Canine	Nakao *et al.*, 2013 (17)
T. ovis	JX134111-JX134114 JX134120-JX134122	Iran	Sheep	Rostami *et al.*, 2019 (23)
T. solium	AB066491	Ecuador	Pig	Nakao *et al.*, 2002 (18)
T. saginata	AB533173	Thailand	Human	Okamoto, 2016 unpublished
T. multiceps	DQ321830	Italy	Sheep	Varcasia *et al.*, 2006 (28)
T. hydatigena	MT086500	Iraq	Sheep	Mohammed and Hama-Soor, 2020 unpublished
Echinococcus granulosus	GQ168811	India	Buffalo	Pan *et al.*, 2016 unpublished

Table 2. Prevalence of *C. ovis* cysts based on sex and age, in sheep (N* = 6,411) from Sulaymaniyah, Iraq

		Sheep			
		Number of examined	Number of infected (%)	95% CI%	P value of X^2
Overall			84(1.31)	0.21–2.41	
Sex	Male	3,438	54(1.57)	0.82–2.32	= 0.062
	Female	2,973	30(1.00)	0.21–1.79	
Age	Young (<1 y)	3,009	25(0.83)	0.33–1.33	< 0.001
	Adult(>1 y)	3,402	59(1.73)	0.96–0.50	

*Total number of examined sheep

Table 3. Frequency distribution of *C. ovis* cysts in sheep (N*= 6,411) from Sulaymaniyah, Iraq

Predilection sites	Number of infected (%)	95% CI%	P value of X^2
Heart muscle	81(1.26)	0.29–2.23	< 0.05
Diaphragm	65(1.01)	0.17–1.85	
Intercostal muscles	55(0.85)	0.19–1.51	
Abdominal muscles	42(0.65)	0.03–1.33	

*Total number of examined sheep

Table 4. Haplotype distribution patterns of *T. ovis* from Iraqi sheep based on mitochondrial COX1 gene with associated accession numbers

Haplotype	Number of specimens (isolates)	GenBank accession No.
IQTOH1	13	MW017206
IQTOH2	8	MW017207
IQTOH3	6	MW017208
IQTOH4	4	MW017209
IQTOH5	3	MW017210
IQTOH6	1	MW017211

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
[1]	T. ovis: AB731675 (New Zealand)																			
[2]	IQTOH1: MW017206	0.01																		
[3]	IQTOH2: MW017207	0.01	0.01																	
[4]	IQTOH3: MW017208	0.01	0.01	0.01																
[5]	IQTOH4: MW017209	0.00	0.01	0.01	0.01															
[6]	IQTOH5: MW017210	0.01	0.01	0.01	0.01	0.01														
[7]	IQTOH6: MW017211	0.01	0.02	0.01	0.02	0.01	0.02													
[8]	T. ovis: JX134111 (Iran)	0.00	0.00	0.01	0.01	0.00	0.01	0.01												
[9]	T. ovis: JX134112 (Iran)	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.00											
[10]	T. ovis: JX134113 (Iran)	0.00	0.01	0.00	0.01	0.00	0.01	0.01	0.01	0.01										
[11]	T. ovis: JX134114 (Iran)	0.00	0.01	0.01	0.01	0.00	0.01	0.01	0.00	0.01	0.00									
[12]	T. ovis: JX134120 (Iran)	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01								
[13]	T. ovis: JX134121 (Iran)	0.00	0.01	0.01	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.00	0.01							
[14]	T. ovis: JX134122 (Iran)	0.00	0.01	0.01	0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.00	0.01	0.01						
[15]	T. hydatigena: MT086500 (Iraq)	0.19	0.20	0.20	0.20	0.19	0.20	0.21	0.20	0.20	0.19	0.19	0.21	0.20	0.19					
[16]	T. multiceps: DQ321830 (Italy)	0.14	0.14	0.14	0.14	0.14	0.14	0.15	0.14	0.14	0.14	0.14	0.15	0.14	0.14	0.19				
[17]	T. saginata: AB533173 (Thailand)	0.15	0.15	0.15	0.16	0.15	0.16	0.16	0.15	0.15	0.15	0.15	0.16	0.16	0.15	0.19	0.07			
[18]	T. solium: AB066491 (Ecuador)	0.14	0.14	0.14	0.14	0.14	0.14	0.15	0.14	0.14	0.13	0.14	0.15	0.14	0.14	0.23	0.14	0.18		
[19]	E. granulosus: GQ168811 (India)	0.27	0.28	0.28	0.27	0.27	0.28	0.27	0.27	0.28	0.28	0.27	0.29	0.28	0.26	0.28	0.45	0.34	0.39	

Fig. 1. Comparison of pairwise nucleotide sequence variations between six haplotypes related to the COX1 gene of *T. ovis* (current study) and other associated taeniids

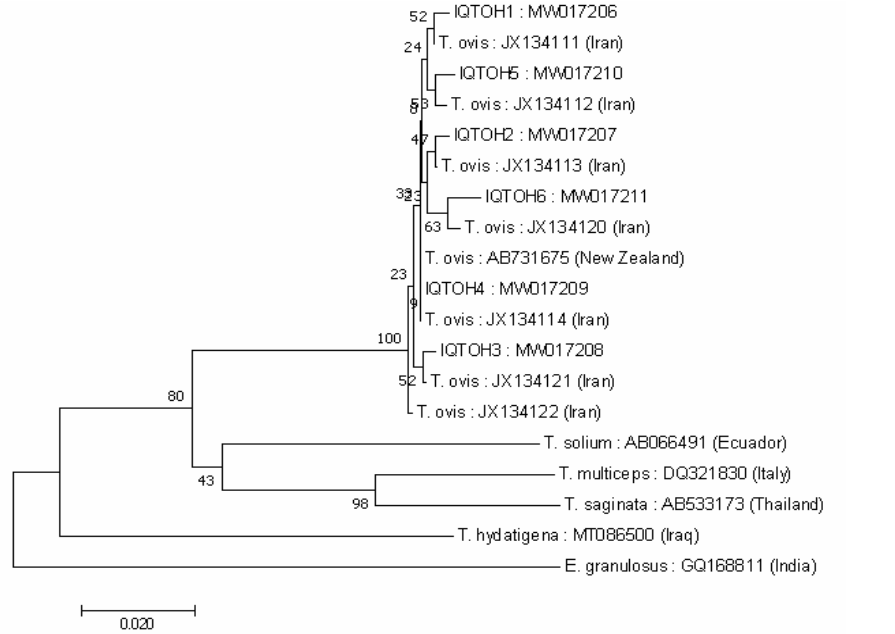

Fig. 2. Phylogeny of sheep *T. ovis* isolates from this work, and other related taeniid sequences. The phylogenetic tree was analysed using the neighbour-joining method from partial mitochondrial COX1 gene sequences. The scale bar shows 2% of variation. MW017206–MW017211 – GenBank accession numbers illustrating *T. ovis* sequences discovered in the presented work; IQTOH1–IQTOH6 – haplotypes of *T. ovis*

Table 5. Nucleic acid substitution and related amino acid changes in the *T. ovis* sheep profiles of six COX1 sequences in Iraq compared to New Zealand (accession number AB731675)

Haplotype (present study)	No. and position of nucleotide substitution	Substitution of amino acid
IQTOH1	225 (T→C)	Tyr →Tyr
	264 (T→C)	Ile →Ile
IQTOH2	163 (T→C)	Phe →Leu
	234 (G→T)	Leu →Leu
IQTOH3	96 (T→G)	Cys →Trp
	183 (C→T)	Ile →Ile
IQTOH4	_*	_*
IQTOH5	223 (T→C)	Tyr →His
	345 (T→C)	Asp →Asp
IQTOH6	144 (T→A)	Asp →Glu
	163 (T→C)	Phe →Leu
	229 (C→T)	Leu →Phe
	352 (C→T)	Leu →Phe

*No substitution

Discussion

In the current study, the overall prevalence of *C. ovis* was 1.31%, which is in accordance with previous reports of *C. ovis* parasitism rates among sheep of 1.27% prevalence in Kermanshah, western Iran (12) and 2.02% in Egypt (1), although it was relatively higher than that registered in Fars province, southern Iran (1%) (20). However, the prevalence

found in this study is lower than those recorded in sheep of Isfahan, Iran (2.9%) (9), eastern Ethiopia (26%) (26), south-west England (7%) (6), Canada (48%) (4), Tasmania (3.4%) (22) and China (58.9%) (25).

In Sulaymaniyah, Iraq, it appears that the recorded lower prevalence of *C. ovis* infection may have been due to a real reduction in infections or less careful examination of the slaughtered sheep in the abattoir (11). Cysticerci of *T. ovis* are easily missed, as they may not be present at the location of the routine incisions, given that most cases of cysticercosis are mild infections. Among the many other contributory factors in accuracy or inaccuracy in finding cysticerci are variations in the skills and motivation of meat inspectors, the speed of the slaughter process, and the standard of the meat inspection facilities (7).

As observed during the present study, several factors may be suggested to contribute to the existence and continuity of infection with *T. ovis* in dogs and its metacestode *C. ovis* in sheep. Sheep wandering the streets, and eating refuse polluted with the faeces of stray dogs might be exposed to many eggs of infectious parasites, including *T. ovis*. Many sheep are slaughtered elsewhere than in facilities carrying out inspections to escape carcass condemnation and charges imposed for inspection, and this practice risks perpetuating the transmission cycle between sheep and dogs. Moreover, guard dogs living with flocks of sheep for their protection from wild carnivores while they graze also play a significant role in transmitting tapeworms from dogs to sheep in rural and urban areas, and *via* these *T. ovis* causing sheep measles (1).

The infection rate with *C. ovis* was higher in male sheep (1.57%) than in female sheep (1%). These findings agree with those of Abdelaziz *et al.* (1), who found that male animals had the higher infection rate (52.63% compared with 47.36%). On the other hand, the results of this study are not in accordance with those of a previous investigation conducted in Iran (9), in which the rate of infection was higher in females. This could be due to the higher numbers of young males slaughtered than females since consumers desire males' meat more, whereas females were kept alive for longer to breed.

In this research, older sheep were more frequently infected, which may be due to the immune system of the animals. This result is considered consistent with the results of the previous research performed in Egypt (1), where the *C. ovis* infection rate was higher in old sheep than young ones. This could be due to long companionship periods with guard dogs throughout the time grazing as adult animals, whereas lambs are often left in buildings.

As for the preferred site of infection for *C. ovis* in the muscles examined, there was a substantial association between the infection rate and the location of the cyst, with the highest proportions in cardiac muscles indicating the parasite's tissue tropism. This could be due to the hexacanth embryo's migration *via* the circulatory system to the caudal vena cava and after that to the heart, in which they settle as the first muscle in which they can live and encyst. The embryos remaining in circulation gradually settle within the abdominal, costal, and diaphragmatic muscles, and the lower proportions of cysts detected in these tissues seem to bear this out. Such results are in accordance with those of various publications of research from around the world (1, 9, 12, 30).

Although, the distribution of this cysticercus in different body parts followed no definite pattern, it appears that muscle activity, animal age and the geographical area where the sheep was herded largely determine the predilection sites in slaughtered animals (16, 19).

COX1 sequence analysis of six *T. ovis* haplotypes in this study indicated 11 nucleotide substitutions, of which two were nucleotide transversions. Table 5 shows the detection of five amino acid changes due to five nucleotide substitutions within the haplotypes. There is no evidence that any change in the amino acid compositions related to cytochrome c oxidase could impact the function of the enzyme or parasite adaptation. However, it was proven in other parasitic species that a single amino acid change could affect the organism's biological fitness (21, 27).

The presented study indicated a fairly low degree of variation in the COX1 gene of *T. ovis* compared to other isolates of this taeniid. In addition, pairwise comparison of the isolates of *T. ovis* from this experiment as well as available mitochondrial sequences from Iranian (23) and New Zealand (17) sheep isolates indicated 0.0–2.0% and 0.0–1.0% nucleotide difference in the COX1 gene, respectively (Fig. 2). Therefore, the phylogram showed that the present research's Iraqi isolates were comparable to other isolates of *T. ovis*, as they share 98.95–99.74% identity with those from New Zealand and Iran.

The dendrogram created by phylogenetic analysis uniformly clustered all the COX1 haplotypes into a single clade together with a *T. ovis* reference sequence (accession number AB731675). As a distinct subclade, other taeniids including, *T. solium*, *Echinococcus granulosus*, *T. multiceps*, *T. saginata*, and *T. hydatigena* were grouped together.

Although the results of this study showed the low prevalence of *C. ovis* infection, there was no data concerning *C. ovis* in the study area. A molecular analysis of the material in the present study revealed five newly recorded haplotypes of *T. ovis*. The tapeworm *T. ovis* and its metacestode *C. ovis* need further epidemiological and molecular research to allow Iraq to improve efficiency in planning for the prevention and control of parasites. By monitoring unlawful slaughter elsewhere than in slaughterhouses and the hygienic disposal of abattoir offal, condemned tissues, and organs, local veterinary authorities should restrict infections among stray dogs. In addition, in hygienic rearing systems, sheep should be kept away from contact with contaminated refuse and food or stray dogs. In order to further the understanding of the nature of the genetic variation in these taeniids, more research is needed to elucidate the mitochondrial and nuclear genes from the isolates of *T. ovis* from various endemic countries worldwide.

Conflicts of interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

Financial Disclosure Statement: The author's own resources.

Animal Rights Statement: This research was performed in compliance with applicable national and international animal handling standards, with care to ensure animal health was respected. All procedures were explained before sampling to abattoir authorities, owners and veterinarians. Ethical approval was obtained from the Ethics Committee of the College of Veterinary Medicine, Sulaimani University.

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