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Original Article

Molecular phylogenetic analysis of *Cysticercus ovis* from Egypt based on *MT-CO1* gene sequences

Análise filogenética molecular de *Cysticercus ovis* do Egito com base nas sequências do gene *MT-CO1*

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Abstract

Cysticercus ovis or sheep measles is the larval stage of *Taenia ovis,* which is the intestinal tapeworm of dogs. It is found in the cardiac and skeletal muscles of sheep and can be the cause of partial or total condemnation of carcasses at abattoirs. The aim of the current work was to determine the prevalence of *C. ovis* among sheep in Upper Egypt and to present the molecular and phylogenetic analysis of this using the amplified Mitochondrial Cytochrome Oxidase subunit 1 (*MT-CO1*) gene. A total of 1885 sheep slaughtered at local abattoirs of 4 different governorates of Upper Egypt (Asuit, Sohag, Qena and Aswan) were carefully examined for *C. ovis*. The overall prevalence of infection was 2.02%. The highest rate of infection was observed in adult animals over 4 years of age (44.73%). There was no significant effect of animal sex on infection rates. The phylogenic analysis of *C. ovis* Egyptian isolates showed very close similarity to the New Zealand isolate (AB731675). This is the first report showing the genetic analysis of *C. ovis* in Egypt, which provides a very powerful tool for taxonomy and definitive diagnosis of *C. ovis,* which could be helpful for preventive and control programs.

Keywords: *Cysticercus ovis*, sheep, prevalence, molecular, phylogeny.

Resumo

Cysticercus ovis "sheep measles" é o estágio larval da *Taenia ovis*, encontrada nos músculos de carneiros, causado pela ingestão de ovos de *Taenia ovis*, parasita de cães. O objetivo do presente trabalho foi determinar a prevalência de *C. ovis* entre ovinos no Alto Egito e apresentar as análises moleculares e filogenéticas, utilizando o gene da subunidade mitocondrial citocromo-oxidase amplificada 1 (*MT-CO1*). Um total de 1885 ovinos abatidos em matadouros locais de 4 províncias diferentes do Alto Egito (Asuit, Sohag, Qena e Aswan) foram cuidadosamente examinados para *C. ovis*. A prevalência geral de infecção foi de 2,02%. A maior taxa de infecção foi observada em animais adultos com mais de 4 anos de idade (44,73%). Não houve efeito significativo do sexo nas taxas de infecção. A análise filogenética de isolados egípcios de *C. ovis* mostrou uma similaridade muito próxima ao isolado da Nova Zelândia (AB731675). Este é o primeiro relato mostrando a análise genética de *C. ovis* no Egito, fornecendo uma ferramenta para taxonomia e diagnóstico definitivo de *C. ovis*, podendo ser útil para programas preventivo e de controle.

Palavras-chave: *Cysticercus ovis*, ovinos, prevalência, molecular, filogenia.

Introduction

In Egypt, the sheep population is estimated at 5.5 million animals (OIE, 2017; SULTAN et al., 2016), raised mainly in either small numbers kept in the household by farmers or in village flocks managed by shepherds (AIDAROS, 2005).

Sheep and goats are the intermediate host for several canine tapeworm species, including *Taenia ovis*. In sheep, the larval stages of this cestode, *C. ovis,* cause cystic lesions in skeletal and heart muscle (SOULSBY; MÖNNIG, 1982).

The habitat of the adult cestode worm *T. ovis* is the intestine of dogs, foxes and wolves. Sheep get the infection through eggs on contaminated pastures or feeding on contaminated garbage

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(DEWOLF et al., 2014). The parasitic cycle is completed after the definitive host eats viable *C. ovis* cysts.

Usually, the specific identification of taeniids has been centered on morphological measures, usually with regards to ecological and biological aspects like host specificity (ABULADZE & SKRJABIN, 1964).

The development of molecular genetic techniques has provided advanced tools for the identification of taeniid species and for investigating relationships among them. In particular, mitochondrial DNA sequencing has been successfully used for the identification and genetic characterization of these parasites (BOWLES & MCMANUS, 1994).

Based on morphology, several studies have been conducted in Egypt to identify *C. ovis* of sheep (DYAB et al., 2017; FAHMI, 2014; OMAR et al., 2016; SULTAN et al., 2010), and very few of these studies focused on the molecular and phylogenetic characterization of *C. ovis* in Upper Egypt.

Parasitic infections have a great economic impact on animal production and causes losses including the retardation of growth, emaciation, and low production of milk, meat and wool. Furthermore, *Cysticercus ovis* in particular is causing economic losses due to partial or total condemnation of the carcass (PERRY & RANDOLPH, 1999; ZHENG, 2016). Therefore the aim of the present study was to determine the prevalence of *C. ovis* among slaughtered sheep in Upper Egypt, and to apply the molecular phylogenetic analysis of *C. ovis* through the use of Mitochondrial Cytochrome Oxidase subunit I (*MT-CO1*) gene sequencing.

Materials and Methods

Ethical considerations

This study followed the institutional ethical and animal care guidelines approved by supreme council of Egyptian universities, Egypt. All procedures were explained prior to sampling to abattoir authorities, veterinarians, and owners. The sheep from which *C. ovis* were collected in selected provinces in Upper Egypt were part of the normal work of the abattoir.

Study area

The samples were collected from local abattoirs of the Upper Egypt governorates Assiut, Sohag, Qena and Aswan over the period from January to December 2017 (Figure 1).

Sample collection and laboratory examination

A total of 1885 samples were included in the study. Samples were collected and labeled with location, age, sex and site of infection.

Slaughtered sheep were subjected to routine meat inspection. The heart, diaphragm, tongue and major skeletal muscles were investigated for the presence of any *C. ovis* cysts by visual inspection, incisions and palpation. The muscles infected with *C. ovis* cyst were transferred to the laboratory of the Parasitology department, Faculty of Veterinary Medicine, Sohag University.

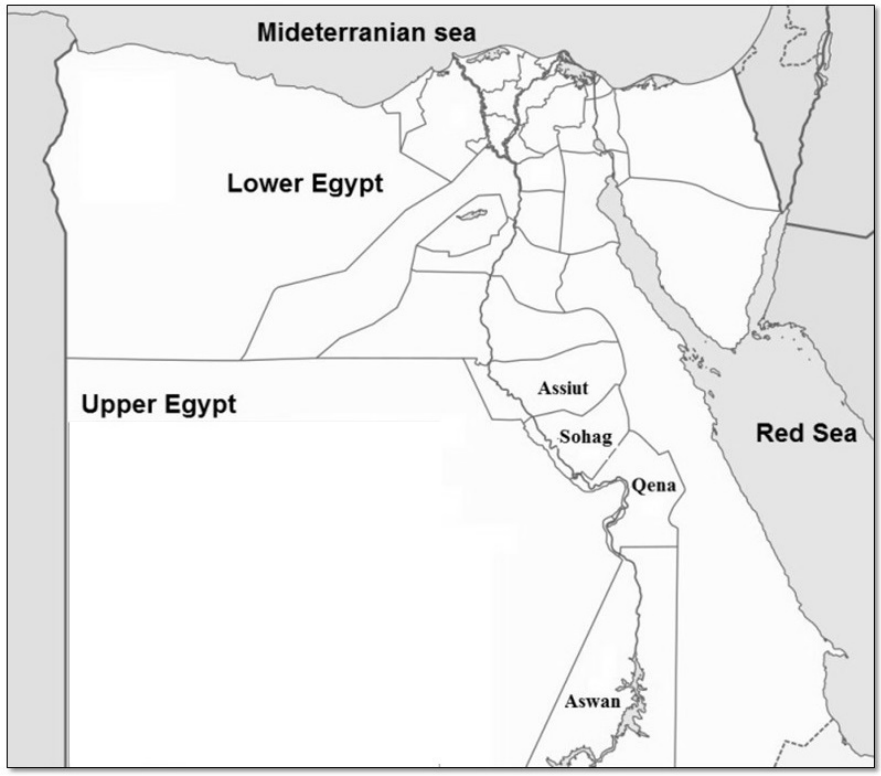

Figure 1. Map of Egypt showing the prevalence of infection of *Cysticercus ovis* among sheep in governorates of Upper Egypt.

Samples were fixed in 10% buffered formalin, stained with alum-carmine and mounted on glass slides for morphological identification (KAUFMANN, 2013; SOULSBY & MÖNNIG, 1982) while other samples were fixed in 70% ethanol for molecular identification.

Molecular characterization

DNA extraction

Approximately 25 *C. ovis* cysts from the infected muscles were pooled and washed in normal physiological saline. DNA extraction was performed once from the 25-pooled cysts using the Qiagen Tissue Kit (Qiagen, Germany) according to the manufacturer's protocol.

PCR, and sequencing of the mitochondrial MT-CO1 gene

The *MT-CO1* gene was amplified by PCR using the following primer pairs: CO1-F 5′-ATGAATATTAAAACTTTATTAAGTTGGA-3′ and CO1-R 5′-TTAAACTAAAAAACCACGGGCA-3′, and this was performed with minor modifications according to methods previously described (Shi et al., 2016). PCR was done with the following conditions: 94 °C for 5 min, then followed by 30 cycles of 94 °C for 1 min, 50 °C for 35 s, 72 °C for 2 min and a final extension at 72 °C for 10 min.

PCR products were observed on a 1.0% (w/v) ethidium bromide stained agarose gel and visualized and photographed on a UV transilluminator. PCR products were directly purified and sequenced using the ABI 3370 DNA sequencer at the Molecular Biology Unit (Animal Health Research Institute, El-Dokky, Egypt) applying a primer walking strategy (JIA et al., 2010).

Assembling of nucleotide sequences and phylogenic analysis

The obtained *MT-CO1* sequences of *C. ovis* were edited in MEGA6 (TAMURA et al., 2013) and Clustal W 12.1 V software, and were also aligned with reference sequences available using BLAST algorithms and databases from the National Center for Biotechnology (NCBI).

The phylogram was constructed using the neighbor-joining method analysis of coding genes in the mitochondrial genome of *T. ovis* based on comparison and alignment with reference sequences of other species in Taeniidae (LAVIKAINEN et al., 2008; SHI et al., 2016) (Figure 2), and the tree was rooted with *C. pisiformis* (GU569096.1) using Kimura's two parameter model. The phylogram is drawn to scale, with branch lengths (next to the

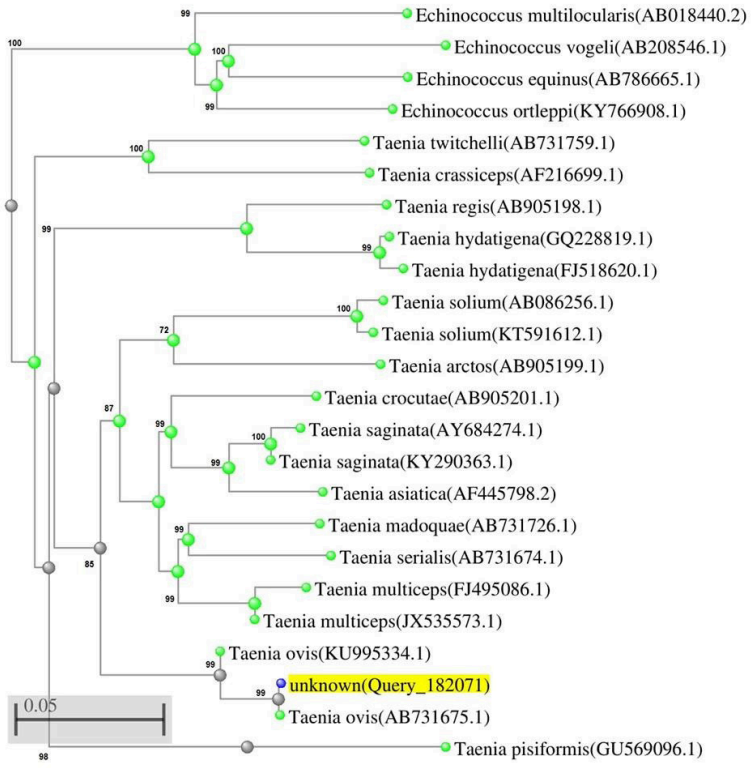

Figure 2. Phylogenetic tree and evolutionary relationship of *Taenia (Cysticercus) ovis* Egyptian isolate (Unknown (Query_182071) based on the *MT-CO1* gene locus using the Clustal program for pair-wise and multiple sequence alignment; the tree was constructed by the neighbor-joining analysis (NJ) with MEGA6 software using *Echinococcus multilocularis* as out group, with genetic distance of 0.05, it was closely similar to *T. ovis* New Zealand isolate with Accession no. (AB731675.1) and (KU995334.1) (TAMURA et al., 2013).

branches) in the same units as those of the evolutionary genetic distances (0.05%) (TAMURA et al., 2013). The double bootstrap method is used in this study to assess the reliability of the statistical analysis and probability of branches distances.

Statistical analysis

The statistical analysis was conducted using the statistical package of Excel in Microsoft Office to calculate prevalence, and SPSS statistics 20 (2002) IBM software to calculate standard error, *P*-value, odds ratio (OR) and confidence interval at 99% (99% CI) to detect risk factors associated with the infection. A statistically significant association between variables was selected if the calculated *P*-value was less than 0.001 ($P < 0.001$).

Results

Prevalence and associated risk factors of C. ovis

The overall prevalence of *C. ovis* infection among 1885 slaughtered sheep in the Assiut, Sohag, Qena and Aswan governorates of Upper Egypt was 2.02% (99% CI: 0.860-1.069). The highest rate of infection with *C. ovis* was 2.7% observed in Qena province, while the lowest rate was 1.49% observed in Aswan province (Table 1, Figure 1).

Statistically, there was no association between the infection rate and the locality of infection and no significant differences between them with a *P*-value of 0.318 (OR: 0.959, 99% CI: 0.860-1.069).

As displayed in Table 1, the highest infection rate, 44.73%, was among sheep with ages of 4 years and above and the age is not a potential risk factor for the infection as the *P*-value was 0.045 (OR: 1.337, 99% CI: 0.260-0.437).

The results (Table 1, Figure 3) show that *C. ovis* appeared as whitish small cyst ranged from 1-1.5 cm diameter and was recovered from cardiac, diaphragmatic, costal and abdominal muscles. Microscopically, it was stained red by Alum-carmine stain and seen as long invaginated protoscolex with a rostellum armed with Taeniidae hooks and four rounded suckers (Figure 4).

Regarding to the site of the infection, the cardiac muscles showed the highest infection rate (34.21%), with a *P*-value less than 0.001, showing a highly significant difference compared to other tissues (OR: 1.370, 99% CI: 1.066-1.761; $P < 0.001$) and a strong association between cardiac muscle and *C. ovis* infection in sheep.

In addition, the infection rate among males (52.63%) was higher than in females (47.36%), as shown in Table 1, without significant difference, where the *P*-value was 0.550 (OR: 0.917, 99% CI: 0.631-1.333). Statistically, there was no association between the animal's sex and the infection rate.

Sequence analysis and phylogeny of C. ovis

The amplified DNA fragment size of the *MT-CO1* gene was nearly 437 bp. The pair wise comparison illustrates the low distance between the sequences from selected *T. ovis* compared with reference sequences from GenBank.

The Egyptian isolate of the current study is highly similar to other *T. ovis* isolates, as it shared 99% identity with the *MT-CO1* gene of the *T. ovis* New Zealand isolate in GenBank AB731675.1 (NAKAO et al., 2013), of China in GenBank KU995334.1 (SHI et al., 2016) and other reference sequences (Figure 2), while

Table 1. Prevalence of *Cysticercus ovis* infection and different associated risk factors in examined slaughtered animals among Assiut, Sohag, Qena and Aswan the provinces of Upper Egypt.

Risk factor		Exam	Inf.	%	S.E.	P-value	O. R. Lower	99% C.I. Upper	
locality	Assiut	418	11	2.63	**0.042**	**0.318**	**0.959**	**0.860**	**1.069**
	Sohag	836	14	1.67					
	Qena	296	8	2.7					
	Aswan	335	5	1.49					
	Total	**1885**	**38**	**2.02**					
Age	1-2 y.	383	7	1.82	**0.101**	**0.0446**	**1.337**	**1.260**	**1.437**
	2-4y.	846	14	1.65					
	Over4y.	656	17	2.59					
	Total	**1885**	**38**	**2.02**					
Site of infection	Cardiac	1885	13	0.69	**0.097**	**0.001***	**1.370**	**1.066**	**1.761**
	Abdominal	1885	8	0.42					
	Costal	1885	6	0.32					
	Diaphragm	1885	11	0.85					
	Total	**1885**	**38**	**2.02**					
Sex	Male	998	20	52.63	**0.145**	**0.550**	**0.917**	**0.631**	**1.333**
	Female	887	18	47.36					
	Total	**1885**	**38**	**2.02**					

99% C.I: Confidence Interval at 99%; S.E: Standard Error; O.R: Odds Ratio; *(significant at P≤0.001).

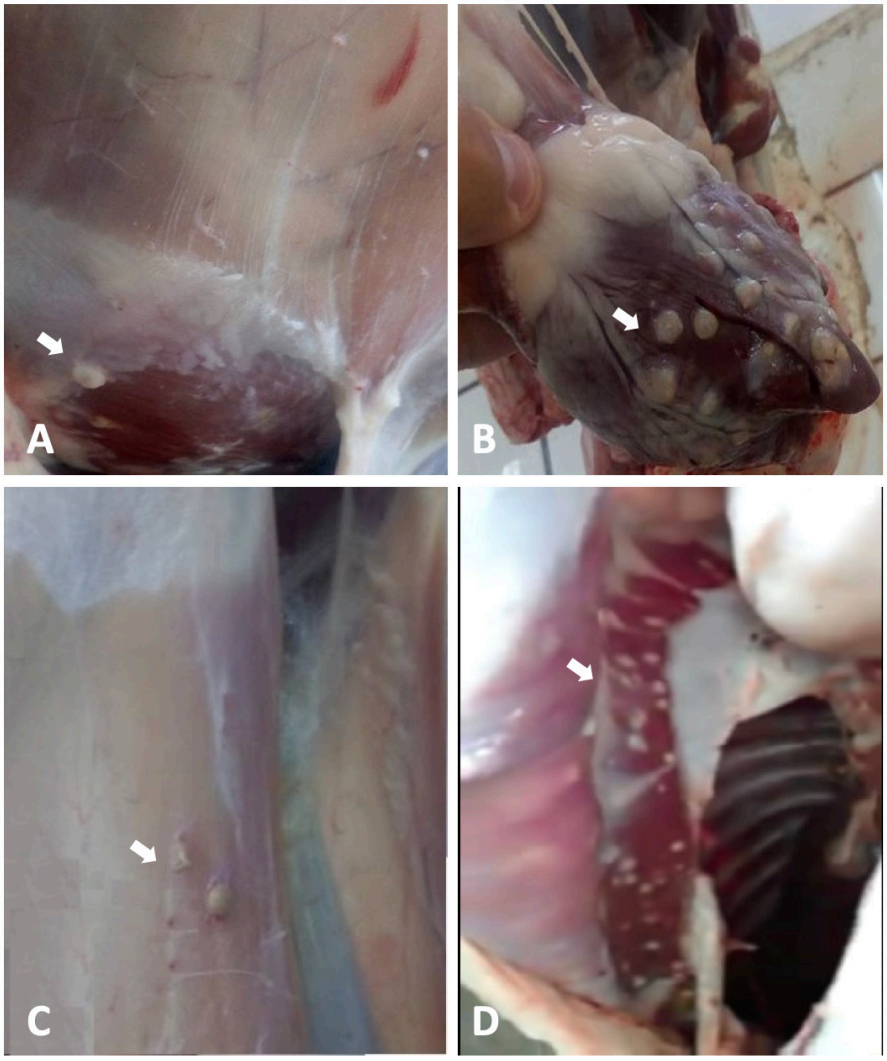

Figure 3. Different muscles of slaughtered sheep infected with *Cysticercus ovis* cyst; (A) *C. ovis* on costal muscles; (B) *C. ovis* on cardiac muscles; (C) *C. ovis* on abdominal muscles and (D) *C. ovis* on diaphragmatic Muscles.

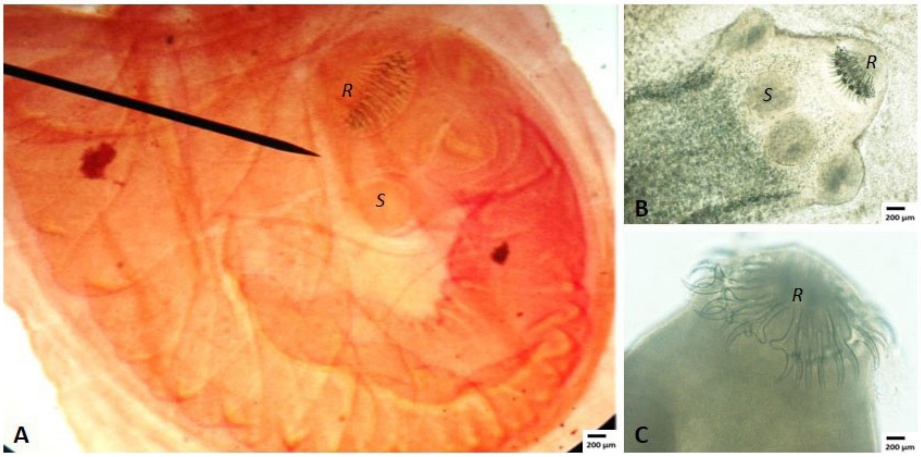

Figure 4. Microscopic examination *Cysticercus ovis* cyst, (A) invaginated protoscolex (stained by carmine), (B) unstained evaginated protoscolex with rostellum and 4 suckers, (C) rostellum with taeniid (X 100). (R) is for Rostellum and (S) is for Sucker.

it shared 75%-91.3% identity with this gene in other *Taenia* species including *T. solium, T. serialis, T. saginata, T. multiceps, T. hydatigena, T. crocutae, T. twittchelli, T. asiatica, T. regis* and *T. madoquae*.

Discussion

The overall prevalence of *C. ovis* in this study was 2.02%, which agreed with the previous studies conducted on *C. ovis* among sheep, with a prevalence of 1.9% in Upper Egypt (DYAB et al., 2017), 2.3% in Saudi Arabia (AL-QUREISHY, 2008) and 1.3% in Iran (HASHEMNIA et al., 2016), although this was relatively higher than those recorded in Qena, Upper Egypt (0.35%) (ALI, 2013) and in Iran (0.1%) (ORYAN et al., 2012).

However, the obtained prevalence is lower than those reported in sheep of Cairo, Egypt (5.7%) (ABEDL-MAOGOOD, 2005), in sheep of Western Australia (20.5%) (WHITE, 1976), in goats of eastern Ethiopia (22%) (SISSAY et al., 2008), in sheep of China (58.9%) (Shi et al., 2016), in sheep of Canada (48%) (DEWOLF et al., 2012) and in sheep of south-west England (7%) (EICHENBERGER et al., 2011).

As observed during doing the current study, several factors can be suggested to contribute the existence and continuity of infection with *T. ovis* in dogs and its metacestode *C. ovis* in sheep. Lost sheep roaming the streets and feeding on garbage which may be contaminated by stool from stray dogs can exposes sheep to many infective parasites eggs such as *T. ovis.* Many sheep are slaughtered daily outside the inspection facilities in order to avoid inspection charges and carcass condemnation, which increases the risk of the infection transmission cycle between dogs and sheep. Moreover, in the rural and urban areas, guardian dogs that are living with the sheep flocks during grazing to protect them from wild carnivores are also playing an important role in the transmission of dog-sheep tapeworms including sheep measles caused by *T. ovis*.

Therefore, preventing the sheep from being exposed to the tapeworm is essential and can be accomplished by deworming the farm or guardian dogs routinely, keeping the stray and wild carnivores away from the sheep grazing areas and hygienic disposal of both condemned carcasses and dead sheep. In addition, prevention and control of slaughter outside the meat inspection facilities by extending these facilities to rural and urban areas and supporting them with well-trained inspectors and veterinarians, as well as diagnostic tools, can further prevent exposure.

The infection rate of *C. ovis* was higher among males (52.63%) than females (47.36%), however without a significant difference ($P < 0.01$) and with no association between animal sex and the infection rate. These results agree with Senlik (2008) and Jayousi (2014) who found that the highest infection rates were observed in male animals (26% and 2.7%, respectively) compared to females (23.9% and 2.15%, respectively).

In contrast our results disagree with other studies that have been done in Egypt (ABU-ELWAFA & AL-ARABY, 2008; DYAB et al., 2017; OMAR et al., 2016), where the infection rates were higher in ewes than rams. This may be due to the higher numbers of slaughtered younger rams in comparison to ewes, as the meat of rams is more favored for consumption by the public, while ewes are preferred and kept alive for longer times for breeding and giving birth.

For different age groups, the effect on infection rates was epidemiologically insignificant. Infection in adults above 4 years old was higher than in younger animals, however without significant differences ($P < 0.01$). This result is in accordance with the results of earlier studies done in Egypt (ABU-ELWAFA & AL-ARABY, 2008; ALI, 2013; DYAB et al., 2017), where the infection rates of *C. ovis* were higher among older adult sheep than in young ones. This may be because of longer time of companionship with the guardian dogs during grazing in adults, while lambs are mostly kept in houses. In addition, the older (over 1-2 years old) and heavier sheep (mostly males) are more favorable for slaughter, and the older females that are finished breeding (mostly over 4 years old) are prepared for slaughter. For all these reasons, the older sheep aged over 4 years old represented a large proportion in the collected samples and subsequently were more highly infected.

As for *C. ovis* preferred site of infection in the examined muscles, there was a significant relationship between the location of the cyst and infection rates, with the highest proportion in cardiac muscles which reflects the tissue tropism of the parasite to the cardiac muscles. This may be due to the migration of the hexacanth embryo through the circulation system to the caudal vena cava, then eventually to the heart where the embryos settled in cardiac muscle, the first muscle of which they can live and encyst inside. The remaining number of circulating embryos will settle subsequently in diaphragmatic, costal and abdominal muscles. These results are in agreement with many previous studies worldwide (DYAB et al., 2017; GESSESE et al., 2015; HASHEMNIA et al., 2016; ZHENG, 2016).

Several earlier studies have been done concerning the molecular identification of *T. ovis* worldwide (DEWOLF et al., 2012; LAVIKAINEN et al., 2008, 2010; SHI et al., 2016). Adding to this, the present study investigated the molecular identity of *T. ovis* in sheep of Upper Egypt and identified the possible origin of the parasite isolate. The results revealed that this isolate is 99% similar to New Zealand isolates. However, it is impossible to determine the original source of the sheep in the country, and the infection might also be transmitted to the local sheep via imported sheep from other countries. Further phylogenetic analysis studies should be carried out in final and intermediate hosts at molecular levels.

Conclusion

The present study elucidates the prevalence of *C. ovis* infection in Egyptian slaughtered sheep at abattoirs. The main associated risk factors for sheep infection with *C. ovis* in the study areas were the age of the animals and the affected muscles (parasite tissue tropism).

C. ovis needs further epidemiological and molecular studies to allow for the country of Egypt to enhance effective planning for parasite prevention and control. Local animal health care authorities are asked to limit the infection among stray dogs by controlling illegal slaughter outside the abattoirs and hygienic disposal of abattoir offals, condemned organs and tissues. In addition to

this, sheep should be kept in hygienic rearing systems away from contact with stray dogs or contaminated foods and garbage.

Obviously molecular diagnosis for *C. ovis* infection significantly helps to differentiate it from such other metacestode and its relation to other taeniid cestodes worldwide, which shows an absolutely different pathogenicity and requires different control programs.

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