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Morphological, Ultrastructural, and Molecular Characterization of *Sarcocystis tenella* from Sheep in Qena Governorate, Upper Egypt

Nermean M. Hussein^{1*}, Amal A. Hassan² Osama H. Abd Ella³

1 - Zoology Department, Faculty of Science, South Valley University, Qena, Egypt

2 - Zoology Department, Faculty of Science, Damanhour University, Egypt

3- Medical Parasitology Department, Faculty of Medicine, South Valley University.

*Email: nermeanmohu@yahoo.com

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ABSTRACT

Small parts of esophagus and cardiac muscles of abattoir sheep from Qena, Upper Egypt between June 2016 to May 2017 were examined by both light microscope (LM), transmission electron microscope (TEM), and DNA sequence analysis of 18 S rRNA gene to determine the rate of infection with *Sarcocystis* spp. and identify the parasite according to morphological, ultrastructure characteristics, and DNA sequence analysis. LM and TEM studies revealed that microscopic, thin walled sarcocysts were detected in 47 of 63 male (74.6 %) and in 32 of 41 female (65.07 %) as a spindle-shaped with size (225 – 431.3 μm x 34.7- 82.4 μm) in esophagus muscles, and ovoid with size (141.6- 263 μm x 51.4- 82.3 μm) in cardiac muscles. The result sequences were compared with other previously sequenced *Sarcocystis* species retrieved from Gene Bank it was most closely referred to *Sarcocystis tenella* (identity 99- 100%).

INTRODUCTION

Sarcocystis is an intracellular protozoan parasite; it may cause fatal disease for its host (Tenter, 1995 & Fayer, 2004). There are four species of *Sarcocystis* infected sheep, two species of macroscopic cysts (*Sarcocystis gigantea* and *Sarcocystis medusiformis*), and the others of microscopic cysts *Sarcocystis tenella* and *Sarcocystis arieticanis* (Collins *et al.*, 1979; O'Donoghue and Ford, 1986; O'Donoghue *et al.*, 1996), the two microscopic species are pathogenic and can lead to death (Dubey *et al.*, 1989), the prevalence of cattle and sheep infections with *Sarcocystis* are between (70-100%) according to (Pereira and Bermejo, 1988; Woldemeskkel and Gebreab, 1996). In addition, *Sarcocystis tenella* was recorded in sheep in Iran and Brazil (Da Silva *et al.*, 2009; Shahbazi *et al.*, 2013). Jehle *et al.*, 2009 identified *Sarcocystis* species according to the structure of its wall.

The aim of this study is to determine the infection rate of *Sarcocystis* in sheep slaughtered in Qena governorate, Upper Egypt -both morphologically and molecularly- as it is the first study in that aspect.

MATERIALS AND METHODS

Parts of esophagus and cardiac muscles of both males and females from ages one to five years slaughtered sheep, were collected from different localities of Qena Upper Egypt and small parts of it were compressed between two glass slides and examined microscopically,

Latif *et al.*, (1999), parts of infected specimens were fixed with 10 % formalin for histological sections that were stained with H & E stain according to Mandour *et al.*, (2011), small parts of infected muscles were fixed in 4% gluteraldehyde for studying by transmission electron microscope, other infected parts were observed in 70% ethyl alcohol for molecular study.

Method of T.E.M:

After specimens fixation with 4% cold gluteraldehyde for 48 hours, washed in cacodylate buffer (PH 7.2) 3–4 times for 20 minutes every time, post fixed in 2% osmium tetra oxide (OSO₄) for 2 hours, then washed in buffer four times, dehydrated by using ascending grades of ethyl alcohol (50–70 – 80–90 and 100% 2 hours for each one), then embedded in epon-araldite mixture (protocol of E.M. unit Assiut Uni, Bozzol and Russell (1991). From the embedded blocks semi thin sections by L K B ultra microtom with thickness of 0.5 µm were prepared, then ultrathin section in thickness of 500 – 700A were made by using leica AG ultramicrotome and contrasted in uranyl acetate and lead citrate, examined under JEM 100 CXII electron microscope at 80 KV and photographed using CCD digital camera Model XR- 41.

Molecular Identification Using 18S Ribosomal Subunit DNA:

Parts of one infected heart and esophagus were kept in ethanol (70%) to be subjected to DNA analysis. After removal of ethanol, genomic DNA was extracted using QIAamp DNA Mini and Blood Mini Kit, QIAGEN according to product manual. We used the following pair of primers Sar-F1 Forward 5'GCACTTGATGAATTCTGGCA3' and Sar-R1 Reverse 5'CACCACCCATAGAATCAAG 3'. (Dalimi *et al.*, 2008) for amplification of 18S ribosomal DNA genes. PCR reactions were done using Taq PCR Master Mix Kit, QIAGEN according to product manual for 25–35 cycles using 9700 Applied Biosystems, Thermo-Fisher. Cycles of PCR were set up, The PCR products were run on a 1.5% agarose gel. Subsequently, the gel was stained by using ethidium bromide, and

appropriate bands were excised and purified with a QIAamp PCR Purification Kit, QIAGEN according to the kit manual. Isolates were then sequenced using forward primer to construct a continuous sequence of the tested DNA. Applied Biosystems 3500 automatic Genetic Analyzer was used. Retrieved sequences were compared and aligned with the database in Gene Bank using the NCBI Basic Local Alignment Search Tool (BLAST). Phylogenetic trees were created using Molecular evolutionary Genetics Analysis Version 6.0. (Mega 6) platform, Tamura *et al.*, 2013, choosing muscle, Edgar, 2004, for multiple alignment, and maximum likelihood tree using Tamura-Nei model for generation of tree with gaps removal. Sequences for other *Sarcocystis* spp. were obtained from Gene Bank, *Trypanosoma cruzi* was used as out-group.

RESULTS

Light and Electron Microscopy

Observations:

Sarcocystis tenella with thin cyst wall were isolated from 79 different organ samples (esophagus and heart muscles) from 104 sheep of both sexes (75.96%), they were examined by light microscope, microscopic sarcocysts in 47 of 63 male animals (74.6%), and in 32 from 41 female animals (65.07%). Using LM, sarcocysts of the esophagus were spindle shaped and measured (225–431.3 µm × 34.7–82.4 µm) as in Figure (1), but sarcocysts of heart were ovoid and smaller in size (141.6 – 263 µm × 51.4–82.3 µm) as in Figure (2), both sarcocysts wall are thick (0.409–0.427 µm), the cysts were septate and their interior compartments were packed with bradyzoites. TEM revealed that the cyst wall had many trapezoidal-like protrusions measuring (0.090–0.011 µm × 0.012–0.013 µm) in size Figure (3b). The ground substances layers lie beneath the primary wall of sarcocysts and its thickness is (0.082–0.088 µm). Fully formed bradyzoite measured (2.01–2.03 µm × 0.45–0.49 µm) Figure (3a): At the base of protrusions there are a small vesicles measured (0.0120–0.0122 µm × 0.012–0.0122 µm) Figure (3b).

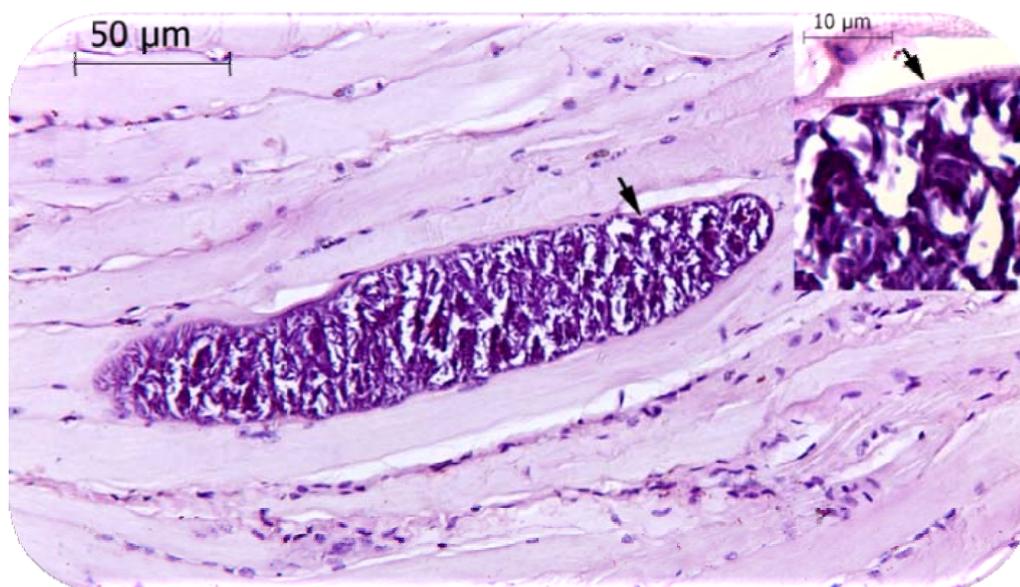


Fig.1: Longitudinal section in oesophagus muscles of sheep infected with *Sarcocystis* sp., the arrow refers to the microscopic *Sarcocystis* sp.

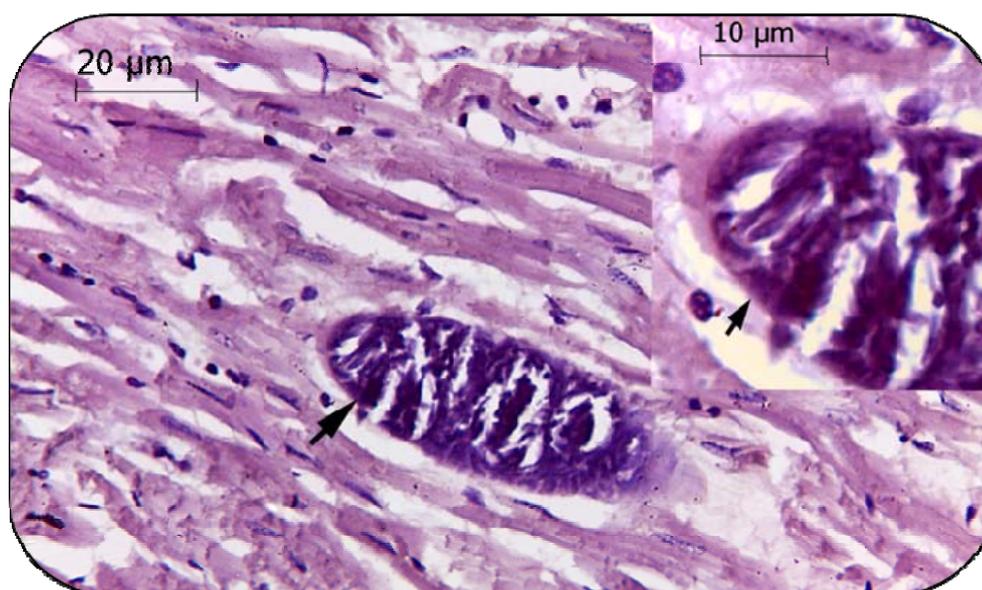


Fig. 2: Longitudinal section in microscopic *Sarcocystis* sp. (black arrow) in cardiac muscles of sheep stained with potassium alum carmine stain.

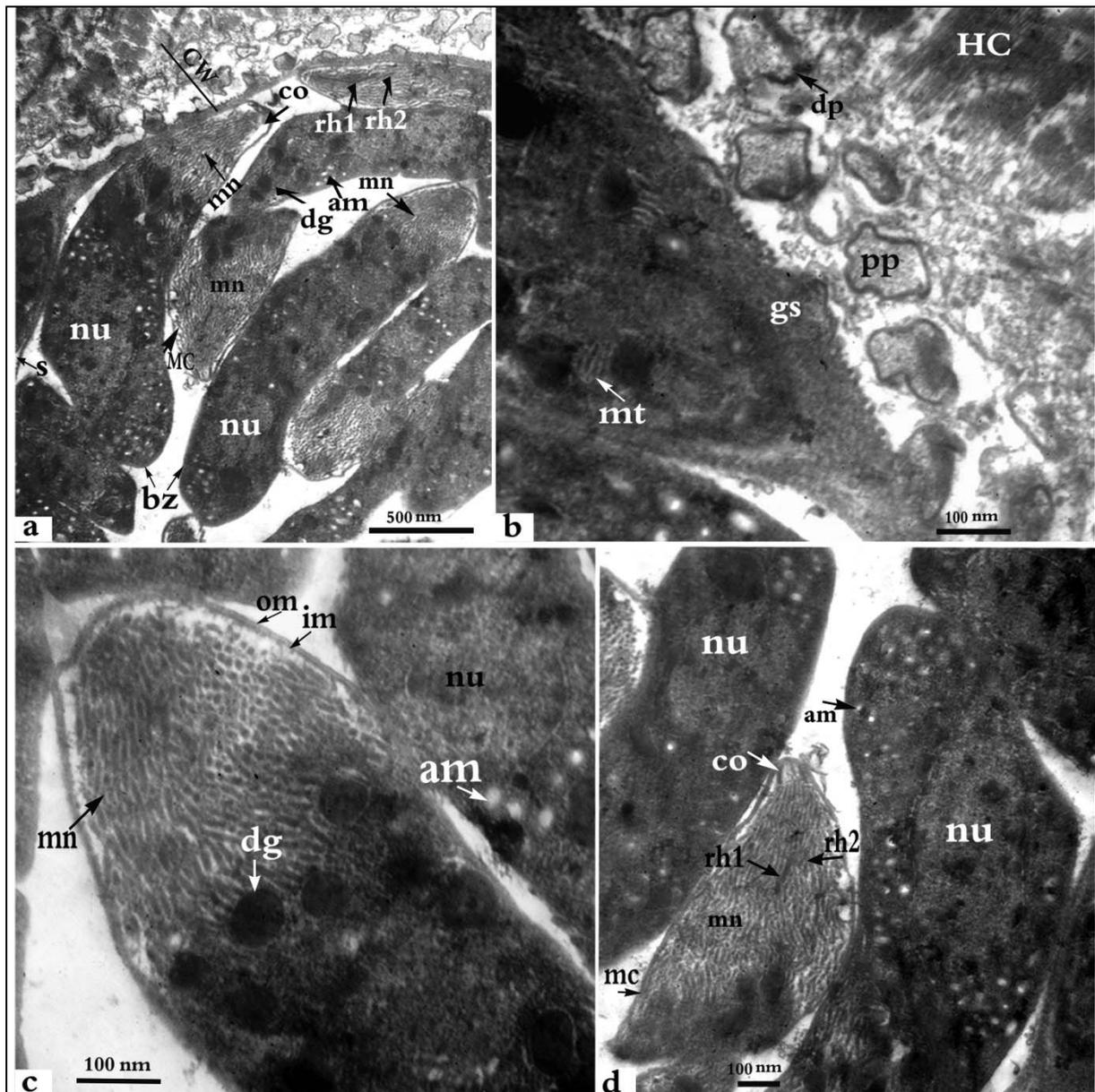


Fig. 3: TEM of *Sarcocystis* sp. from esophagus muscles of infected sheep, **a-** showed the cyst wall thickness (CW), bradyzotes (bz), metrocytes (MC), septa (S), nucleus (nu), micronemes (mn), dense granules (dg), conoid (co), amylopectin (am), and rhoptries (rh). **b-** High magnification part showed the palisade-like protrusions (pp), dense plaques (dp), ground substance (gs), host cell (HC), and the mitochondrion inside the metrocyte. **c-** Enlarged part of mature bradyzoites showed the outer membrane (om), inner membrane (im), micronemes (mn), dense granules (dg), nucleus (nu), and amylopectin (am). **d-** High magnification of metrocyte (mc) showed the two rhoptries (rh1 & rh2).

Sequencing and Genotyping of Isolates:

Sequencing and genotyping of isolates the partial 18S rRNA gene of *Sarcocystis* species was amplified at a specific 600 bp band on the agarose gel Figure (4). The results obtained from sequencing of esophagus sample 1 and heart muscles 2 were compared with available *Sarcocystis* DNA sequences in the Gene Bank based on sequence analysis of 18s r RNA region using BLAST software program, and maximum likelihood tree using Tamura-Nei model for generation of tree with gaps removal. It was found that the genotype of present microscopic cysts Figure (5) corresponds to

Sarcocystis tenella and submitted to the Gene Bank under the accession number KP263759.1. based on the sequence analysis of 18S rRNA region using BLAST software program. The two isolates had a similarity of 99% and 99% coverage to *Sarcocystis tenella* isolate 8 accession number KP263759.1 Phylogenetic trees Figure (6) were created using Mega 6 platform (Tamura *et al.*, 2013), choosing MUSCLE (Edgar, 2004) for multiple alignment, sequences for other *sarcocystis* spp. were obtained from Gene Bank, *Trypanosoma cruzi* was used as out-group.

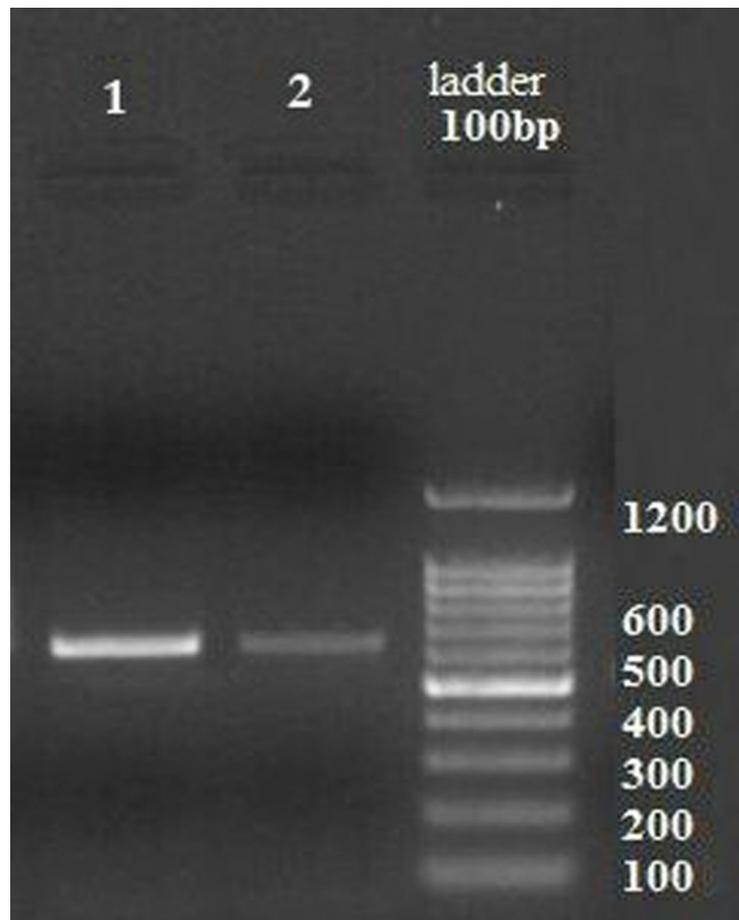


Fig. 4: Gel electrophoresis of 1% agarose gel stained with ethidium bromide for DNA extraction of *Sarcocystis* sp. in different muscles, 1 esophagus and 2 cardiac muscles. Molecular weight marker (100bp).

Range 1: 725 to 1283 [GenBank](#) [Graphics](#) ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
1026 bits(555)	0.0	558/559(99%)	1/559(0%)	Plus/Plus
Query 1	TCGGTG-AAGCCGAGTATCCTGTTTTCCGATTATTGGGATACTGTTACTTTGAGAAAATT	59		
Sbjct 725	TCGGTGAAAGCCGAGTATCCTGTTTTCCGATTATTGGGATACTGTTACTTTGAGAAAATT	784		
Query 60	AGAGTGTGTTGAAGCAGGCTAATTGCCTTGAATACTGCAGCATGGAATAACAATATAGGAT	119		
Sbjct 785	AGAGTGTGTTGAAGCAGGCTAATTGCCTTGAATACTGCAGCATGGAATAACAATATAGGAT	844		
Query 120	TTCGGTTCTATTTTGTGGTTTCTAGGACTGAAATAATGATTAATAGGGACAGTTGGGGG	179		
Sbjct 845	TTCGGTTCTATTTTGTGGTTTCTAGGACTGAAATAATGATTAATAGGGACAGTTGGGGG	904		
Query 180	CATTCGTATTTAACTGTCAGAGGTGAAATTCCTAGATTTGTTAAAGACGAACTACTGCGA	239		
Sbjct 905	CATTCGTATTTAACTGTCAGAGGTGAAATTCCTAGATTTGTTAAAGACGAACTACTGCGA	964		
Query 240	AAGCATTGCGCAAGAGATGTTTTCTAATAATCAAGAACGAAAGTTAGGGGCTCGAAGACGAT	299		
Sbjct 965	AAGCATTGCGCAAGAGATGTTTTCTAATAATCAAGAACGAAAGTTAGGGGCTCGAAGACGAT	1024		
Query 300	CAGATACCGTCGTAGTCTTAACCATAACTATGCCGACTAGAGATAGGAAAATGTCATTT	359		
Sbjct 1025	CAGATACCGTCGTAGTCTTAACCATAACTATGCCGACTAGAGATAGGAAAATGTCATTT	1084		
Query 360	TGCTGACTTCTCCTGCACCTTATGAGAAATCAAAGTCTTTGGGTTCTGGGGGGAGTATGG	419		
Sbjct 1085	TGCTGACTTCTCCTGCACCTTATGAGAAATCAAAGTCTTTGGGTTCTGGGGGGAGTATGG	1144		
Query 420	TCGCAAGGCTGAAACTTAAAGGAATTGACGGAAGGGCACCACCAGGCGTGGAGCCTGCGG	479		
Sbjct 1145	TCGCAAGGCTGAAACTTAAAGGAATTGACGGAAGGGCACCACCAGGCGTGGAGCCTGCGG	1204		
Query 480	CTTAATTTGACTCAACACGGGGAAACTCACCAGGTCAGACATGGGAAGGATTGACAGAT	539		
Sbjct 1205	CTTAATTTGACTCAACACGGGGAAACTCACCAGGTCAGACATGGGAAGGATTGACAGAT	1264		
Query 540	TGATAGCTCTTTCTTGATT	558		
Sbjct 1265	TGATAGCTCTTTCTTGATT	1283		

Fig. (5): DNA sequence alignment of 18S rRNA gene derived from the comparative analysis of the Egyptian *Sarcocystis tenella* with the published sequences of *Sarcocystis tenella* isolate 8 on gene bank (accession No. KP263759.1 showing sequence matches and gaps.

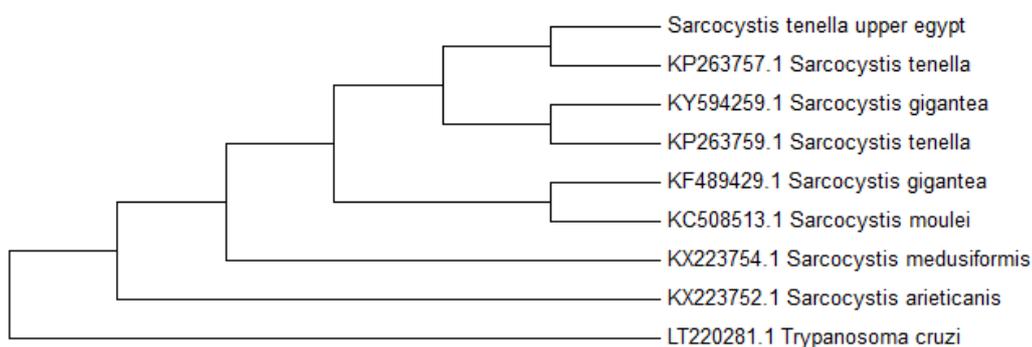


Fig. (6): Phylogeny of *Sarcocystis* spp. isolates by the program Mega v.6. using maximum likelihood method and based on 18S rRNA gene. The reference sequences accession numbers are included.

DISCUSSION

From the previous studies throughout the world, there were different species of *Sarcocystis* recorded in different animals (Gjerde 1985, Da Silva & Langoni 2009, Nourani *et al.*, 2010 & Shahbazi *et al.*, 2013). Gjerde, 1985 isolated and characterized *Sarcocystis grueneri* from reindeer based on molecular method, Al-Hoot *et al.*, 2005 isolated *Sarcocystis moulei* from the sheep in Saudi Arabia, Da Silva and Langoni 2009 recorded *Sarcocystis tenella* from sheep in Brazil and Rafał *et al.*, 2015 recorded and identified *Sarcocystis tenella* by molecular method from Tatra chamois (*Rupicapra rupicapra tatraica*) in Poland. *Sarcocystis gigantea* and *Sarcocystis arieticanis* were recorded from the sheep by PCR-RFLP method in Qazvin province, Iran (Dalimi *et al.*, 2008), and also *Sarcocystis tenella* recorded from sheep in Iran (Shahbazi *et al.*, 2013). Additionally, Mahran 2009 indicated macroscopic (*Sarcocystis gigantea*) and microscopic (*Sarcocystis tenella*) cysts morphometrically in Egypt. Pereira and Bermejo, 1988 and Pejman *et al.*, 2014 identified Macroscopic and microscopic sarcocysts that were isolated from sheep molecularly in Iran.

The rate of infection with *Sarcocystis* sp. from sheep in present study is (75.96%) and this agrees with Woldemeskel and Gebreab, 1996. The cyst walls of the present Sarcocysts are thick and their shape and measurements were agreed with *Sarcocystis tenella* in Polish tatra chamois according to Kolenda *et al.*, (2015), Hong *et al.*, (2016) and the shape of protrusions, the ground substance layer thickness of the present are similar to the *Sarcocystis tenella* from sheep (Hu *et al.*, 2017), but differ than *Sarcocystis arieticanis* Hu *et al.*, 2017 that had a thin cyst wall with a hair-like protrusions.

Sarcocystis tenella can lead to acute sarcocystosis in the infected sheep (Heckerth and Tenter 1998). Non-specific infection symptoms, anorexia, tachycardia, and anemia could be observed following the infection with this parasite. In acute

sarcocystosis, it can lead to encephalitis, encephalomyelitis and subsequently death for the infected sheep (Munday 1984 and Jeffrey 1993). Chronic sarcocystosis reduced meat, milk, and wool and that create economic problems (Collind *et al.*, 1976-Munday 1984). Also, *Sarcocystis tenella* produces microscopic cyst which is invisible so that it remain and causes economic losses. *Sarcocystis* spp. are common in sheep, goats, cattle, camels, birds, and others. In present study, we used light microscopy, TEM revealed that both of esophagus and heart sarcocysts are similar in thin cyst wall, shape, bradyzoites, and merozoite structures, presence of septa that divide the cyst into many compartments, ground substances and the presence of small vesicles at the base of the protrusions, size, and shape of protrusions. According to (Heckerth and Tenter, 1999 and Yang *et al.*, 2001), the most successful method for identification of *Sarcocystis* species is the analysis of variable regions of 18S rRNA gene, molecular studies on *Sarcocystis* species are not widely performed on sheep in Egypt. Therefore, the current study was undertaken to identify the *Sarcocystis* sp. that isolated from different parts of esophagus and cardiac muscles of abattoir sheep in Qena, Upper Egypt by PCR and sequence analysis methods and identifying it as *Sarcocystis tenella*.

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