

Morphological and Molecular characterization of *Sarcocystis arieticanis* from the Heart Muscles of Domestic sheep, *Ovis aries*, in Qena, Upper Egypt

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ABSTRACT

This study examined *Sarcocystis* spp., cyst forming coccidians whose intermediate hosts include sheep, an important source of meat in Egypt. Samples of heart muscles were collected from 110 sheep from different localities in Qena, Upper Egypt, and examined for *Sarcocystis* spp. by macroscopic evaluation, light microscopy, transmission electron microscopy, and molecular tests. All the sarcocysts found [in 52 of 110 sheep (47.27 %)] were thin-walled (0.32–0.44 μm) and measured 78.56–146 μm in length and 33.12–73 μm in width. The cyst walls possessed irregularly branched protrusions (0.67–1.4 $\mu\text{m} \times$ 0.05–0.26 μm) with rounded vesicles at their base that were underlain by a thin layer of ground substance (0.14–0.26 μm). Septa derived from the ground substance divided the cysts into many compartments that contained cystozoites of different developmental stages and fully formed banana-shaped bradyzoites, for which descriptions are provided based on transmission electron microscopy. Comparison of the 18S rRNA sequence of the sarcocysts against those of previously identified species in mammals deposited in GeneBank was performed. The phylogenetic tree clustered the *Sarcocystis* specimens in this study within the *S. arieticanis* clade. In addition, the macroscopic *Sarcocystis* spp. (*S. moulei* and *S. gigantea*) aggregated together at some distance from the microscopic *Sarcocystis* spp. (*S. capracanis*, *S. hiricanis*, *S. tenella*, and *S. arieticanis*) regardless of differences in the intermediate host, indicating the importance of *Sarcocystis* cyst size as a characteristic taxonomic feature.

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Introduction

Sarcocystis spp. are apicomplexan protozoa with an obligatory heteroxenous life cycle, which alternates between an intermediate host and a definitive host (Dubey *et al.*, 1989). They are common parasites of a broad range of vertebrates including mammals, birds, and reptiles (Heydorn and Mehlhorn, 1987; Dubey *et al.*, 1988; Bashtar *et al.*, 1990; Abdel-Ghaffar *et al.*, 1990a; Abdel-Ghaffar *et al.*, 1990b; Abdel-Ghaffar and Al Johany, 2002; Hazirolu *et al.*, 2003; Abdel-Ghaffar *et al.*, 2009; Olias *et al.* 2010; Xiang *et al.* 2010; Mandour *et al.*, 2011; Morsy *et al.*, 2011; Abdel-Baki *et al.*, 2012).

Sheep (*Ovis aries*) are the intermediate hosts of at least six named species of *Sarcocystis*, including *S. tenella* (syn. *S. ovicanis*), *S. gigantea* (syn. *S. ovifelis*), *S. arieticanis*, *S. medusiformis*, *S. mihoensis*, and *S. microps* (Hu *et al.*, 2017), all of which are morphologically differentiated based on their sarcocyst wall ultrastructure. *S. tenella* and *S. arieticanis* produce microscopic sarcocysts transmitted by canids, while *S. gigantea* and *S. medusiformis* produce macroscopic cysts transmitted by felids (Dubey *et al.*, 2016). The remaining two species,

S. mihoensis and *S. microps*, transmitted by canids, are unusual or rare. *S. mihoensis*, reported only from Japan, produces macroscopic sarcocysts (Saito *et al.*, 1997), while *S. microps*, reported only once from China, produces microscopic sarcocysts (Wang *et al.*, 1988). Ultrastructural study of the cyst wall has been used for the identification of *Sarcocystis* spp. isolated from different animals by many researchers (Mehlhorn and Heydorn, 1979; Dubey *et al.*, 1989; Abdel-Ghaffar *et al.*, 2009; Jehle *et al.*, 2009).

Infection with *Sarcocystis* may prove fatal to the host (Tenter, 1995; Fayer, 2004). Hazirolu *et al.* (2003) recorded *S. arieticanis* from cardiac muscles of sheep and were able to describe its wall with the use of transmission electron microscopy (TEM), while Al-Quraishy *et al.* (2014) and Mehlhorn *et al.* (2014) detected *S. arieticanis* from the heart muscles of domestic sheep based on both light and electron microscopy. Bittencourt *et al.* (2016) observed *S. tenella* and *S. arieticanis* in tongue muscles of sheep in Brazil. Hussein *et al.* (2018) identified *S. tenella* in esophagus muscles of sheep in Qena, Upper Egypt using transmission electron microscopy (TEM) and molecular techniques. As part of an ongoing study on *Sarcocystis* spp. infecting sheep in Qena, Upper Egypt (Hussein *et al.*, 2018), a detailed morphological redescription and molecular characterization of *S. arieticanis* collected from sheep heart muscles are presented in this study.

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Materials and methods

Sampling

Specimens were collected from the heart muscles of 110 sheep from different slaughterhouses in Qena, Upper Egypt (93 males and 17 females; aged 6 months to 3 years) during the period from April 2018 to March 2019. The tissues were transported to the laboratory and were examined for Sarcocysts.

Ethical approval was granted by the research and Ethics committee of the Faculty of Science, South Valley University for animal use and collecting permits.

Macroscopic examination

Macroscopic examination was performed by using a hand lens whereas muscle samples was cut into smaller pieces for the presence of macrocysts, which appeared whitish with different shapes according to Lam *et al.* (1999).

Microscopic examination of fresh tissue

Small pieces of heart muscles from each sample were pressed between two glass slides and examined using a light microscope according to Hu *et al.* (2017).

Histological examination

Small pieces of infected heart muscle were fixed in 10% buffered formalin, prepared for 5- μ m paraffin sections, stained with hematoxylin eosin (HE) stain for histological examination and photographed according to Mandour *et al.* (2011).

Transmission electron microscopy

Small pieces of infected heart muscles of one specimen were fixed in 3% cold glutaraldehyde solution in sodium cacodylate buffer 0.1 M (pH 7.4) for 24 h, washed in sodium cacodylate buffer 0.1 M (pH 7.4), postfixed in 1% osmium tetroxide, dehydrated with ethyl alcohol, then embedded in resin. Semi-thin sections were examined by light microscopy. Ultra-thin sections were examined under JEM 100 CXII electron microscope at 80 KV and photographed using CCD digital camera Model XR-41 (Hussein *et al.*, 2018), then the developmental stages were drawn for the first time in present study.

Molecular characterization

Infected heart muscle tissues were cut into small pieces and were preserved in ethyl alcohol 95% at 20 °C for total genomic DNA extraction. DNA was extracted using a DNA Extraction Kit (Qiagen, DNeasy® QIAamp DNA Mini, and Blood Mini Kit). PCR amplifications were performed with a total volume of 50 μ l consisting of 2 μ l of DNA template, 25 μ l PCR master mix of concentration (1 \times PCR buffer, 16 mM (NH₄)₂SO₄, 67 mM Tris-HCl at pH 8.8, 2 mM MgCl₂, 200 μ M of each dNTP, 2 μ M taq DNA polymerase), 0.7 mM each primer and 0.7 unit BIOTAQ™ DNA polymerase (Bioline Ltd.), made up to 50 μ l with Invitrogen™ ultraPURE™ distilled water.

Partial fragments of the 18S rRNA gene (~700 bp) were amplified using the primers 2L (forward: 5'-CGCAAATTACC-CAATCCTGA-3') and 3H (reverse: 5'-ATTCTCATAAGGT-GCAGGAG-3') under the thermocycling conditions specified by Yang *et al.* (2002) as follows: denaturation at 95 °C for 4 min followed by 35 cycles of amplification (94 °C for 30 s, 59 °C for 30 s, 72 °C for 1 min) and a final extension step at 72 °C for 5 min. PCR amplicons were purified using a QIAamp PCR

Purification Kit (QIAGEN) according to the manufacturer's protocol and were sequenced directly using an ABI Prism Big Dye Terminator V.3.1 Cycle Sequencing Kit on an Applied Biosystems 3500 automatic Genetic Analyzer following the manufacturer's recommendations using the same PCR primers. Forward and reverse PCR amplicon reads were used for sequence assembly and editing as recommended by Blasco-Costa *et al.* (2016). GeneStudio™ Professional Edition V. 2.2.0.0 was used to assemble sequences, edit contiguous sequences, and generate consensus sequences.

Newly generated partial 18S rRNA sequences were aligned with sequences of species within the genus *Sarcocystis* and selected outgroup taxa available in GenBank. Sequences were aligned using MUSCLE (Edgar 2004) as implemented in MEGA7 (Kumar *et al.*, 2016) and trimmed to a point where the majority of sequences had started and finished. Two sets of data were obtained, phylogenetic analysis was performed for each one with maximum likelihood (ML), and a best nucleotide substitution fit model was determined using an implemented tool in MEGA7 (Nei and Kumar 2000) where the Akaike Information Criterion indicated that (T92+G+I) was the best estimator for ML settings in both data sets. The bootstrap consensus tree was inferred from 1000 replicates and branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test is shown next to the branches except for bootstrap values less than 50%. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood approach and then selecting the topology with superior log likelihood value.

Results

Morphological data

Macroscopic evaluation revealed no infection with macroscopic sarcocystis in the heart muscles of the domestic sheep. Light microscopy revealed the presence of thin-walled septate microscopic sarcocysts in 52 out of 110 sheep (47.27%), comprising 41 of 93 males (44.08%) and 11 of 17 females (64.70%). The rate of infection in sheep aged over 1 year was greater than that in sheep aged under 1 year. Infection was found in 32 of 58 males (55.17%) aged over 1 year, and in 9 of 35 males (25.71%) aged under 1 year. Infection was found in 9 of 11 females (81.8%) aged over 1 year, and in 2 of 6 females (33.33%) aged under 1 year (Tables 1 and 2). Cyst length was 78.56–146.21 μ m, and cyst width was 33.12–73.54 μ m, (n = 35) (Fig. 1). TEM showed that the cyst wall was thin (0.32–0.44 μ m) and contained horizontally branched protrusions (0.67–1.40 \times 0.05–0.26 μ m; n = 40) with rounded vesicles (0.051–0.066 \times 0.046–0.066; n = 40) at their base underlain by a thin layer of ground substance (0.14–0.26 μ m) located directly under the primary cyst wall (Figs. 2, 4). Several septa derived from the granular layer divided the cyst into many compartments that contained an abundance of cystozoites at different developmental stages.

Table 1. Number of male and female sheep examined and the rate of infection with microscopic *Sarcocystis* sp.

| Gender | No. examined | No. infected | Rate of infection (%) |
|---------|--------------|--------------|-----------------------|
| Males | 93 | 41 | 44.08 |
| Females | 17 | 11 | 64.7 |
| Total | 110 | 52 | 47.27 |

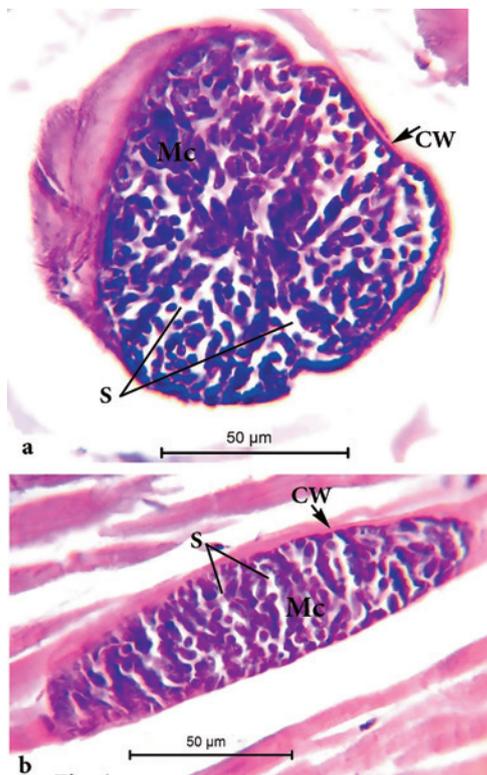


Fig. 1

Fig. 1. Photomicrographs of (a) transverse and (b) longitudinal histological paraffin sections of heart muscles of sheep infected with *Sarcocystis* sp. stained with H&E showing the cyst wall (CW), merozoites (Mc), and septa (S) that divide the cyst into many chambers.

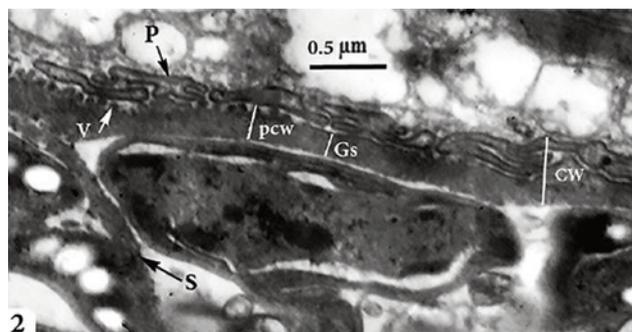


Fig. 2. Transmission electron micrographs of cross sections through a sarcocyst in cardiac muscles of sheep. The cyst wall (CW), irregular horizontally branched protrusions (P), ground substance (GS) that extend into septa (S), and vesicles in the base of protrusions (V).

Developmental stages of bradyzoites

The youngest stage was ovoid with a pointed anterior end (1.2–1.8 × 1.7–2.0 μm; n = 20) filled with micronemes; no other organelles were seen (Figs. 3A-D and 5a). The second stage was pyriform in shape (0.71–0.86 × 2.0–2.5 μm; n = 20), with a small conoid in its anterior end and a large number of micronemes (Figs. 3C, D and 5b). The third stage was also pyriform but larger (1.4–1.8 × 3.1–3.8 μm; n = 20) with conoids, rhoptries, amylopectin and many micronemes (Figs. 3d, 5c). The fourth stage was irregularly-shaped (2.0–2.4 × 3.4–3.8 μm; n = 20) with conoids, rhoptries, amylopectin, and dense granules, but with fewer micronemes than in all the previous stages (Figs. 3C and 5d). The fifth stage was elongate banana-shaped

Table 2. Number of sheep examined and the rate of infection by age

| | No. of examined sheep aged over 1 year | No. of infected sheep aged over 1 year | % | No. of examined sheep aged under 1 year | No. of infected sheep aged under 1 year | % |
|--------|--|--|-------|---|---|-------|
| Male | 58 | 32 | 55.17 | 35 | 9 | 25.71 |
| Female | 11 | 9 | 81.8 | 6 | 2 | 33.33 |
| Total | 69 | 41 | 59.42 | 41 | 11 | 26.82 |

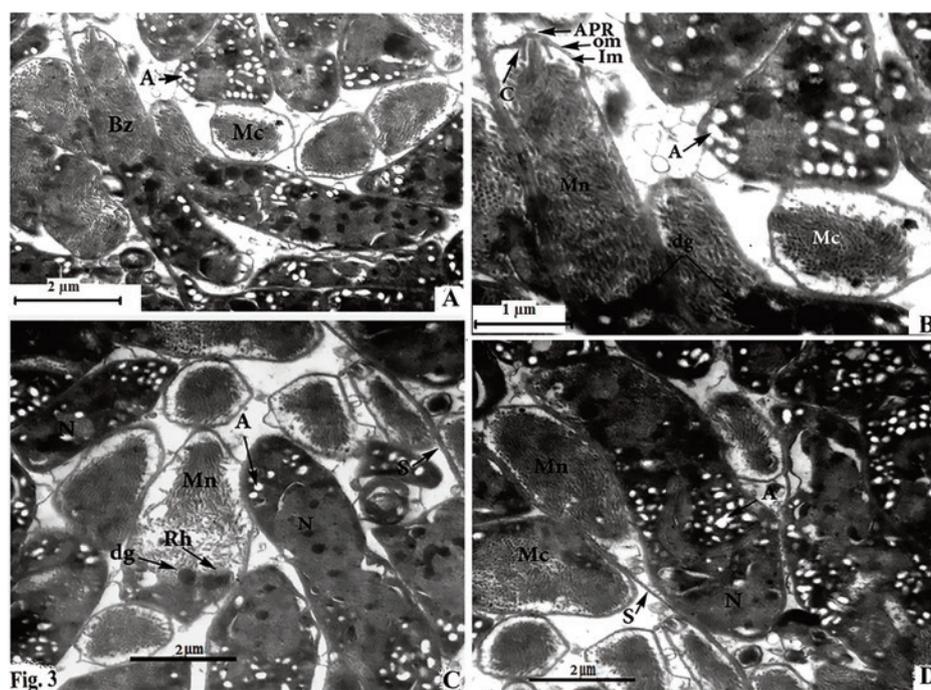


Fig. 3

Fig. 3. Electron micrographs of cross sections through a sarcocyst in cardiac muscles of sheep A: Fully formed bradyzoites with apical polar ring (APR), conoid (C), outer membrane (OM), inner membrane (IM), micronemes (MN), dense granules (dg), and several amylopectin (A), developing bradyzoite (Mc), and nucleus (N) B: Enlarged anterior part of fully formed bradyzoites. C and D: Fine globular metrocyte with micronemes (MN), rhoptries (Rh), dense granules (dg), nucleus (N), and several amylopectin (A).

(2.0–2.4 × 5.2–7.1 μm; n = 20) containing a conoid, rhoptries, micronemes, amylopectin, dense granules, a nucleus and mitochondria (Figs 3D and 5e). The sixth stage, fully formed banana-shaped cystozoites, was surrounded with an inner and

an outer membrane (1.5–2.3 × 6.1–8.5 μm; n = 20) containing all the organelles characteristically found in other *Sarcocystis* spp. (Figs. 3A, 5f).

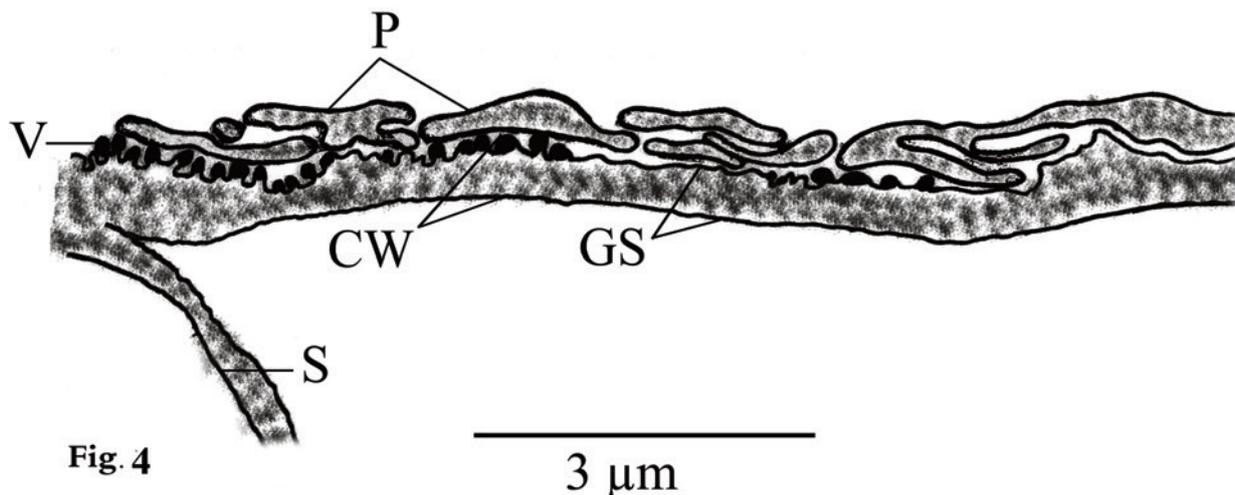


Fig. 4

Fig. 4. Line drawing of thin cyst wall (CW) of present *Sarcocystis arieticanis*. Note protrusions (P), vesicles (V), smooth ground substance (GS), and septa (S).

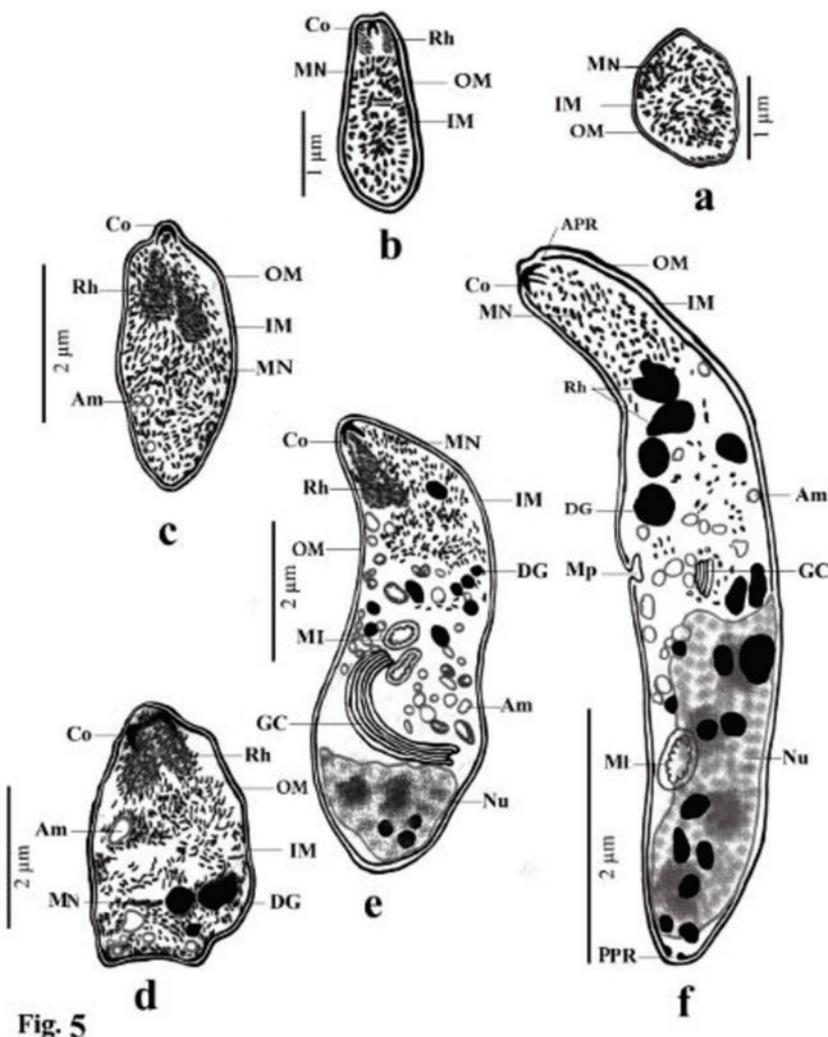


Fig. 5

Fig. (5a–f). Line drawings of different developmental stages of cystozoite of present *Sarcocystis arieticanis* from heart muscles of domestic sheep. Note outer membrane (OM), inner membrane (IM), micronemes (MN), conoid (CO), rhoptries (Rh), anterior polar ring (APR), posterior polar ring (PPR), dense granules (DG), amylopectin (Am), micropore (MP), mitochondria (MI), Golgi complex (GC) and nucleus (N).

arieticanis, *S. hircicanis*, *S. capracanis*, and *S. tenella*. The present *S. arieticanis* resolved within a well-supported clade (56%) representing taxa of *S. arieticanis*. *Sarcocystis hircicanis* resolved in a basal position to all *S. arieticanis* taxa forming a well-supported clade (51%) which in turn was a sister to the strongly-supported *S. capracanis*/*S. tenella* clade (78%).

Discussion

Sarcocystis spp. are among the most common parasites in domestic ruminants, and some of them can generate significant economic losses when causing clinical and subclinical disease. Up till now, at least six species of *Sarcocystis* have been named in sheep; however, only four (*S. tenella*, *S. arieticanis*, *S. gigantea*, and *S. medusiformis*) have been frequently found in different areas (Dubey et al., 2016). *Sarcocystis* spp. have the ability to invade different organs of their intermediate host (Bashtar et al., 1992; Abdel-Ghaffar et al., 2009). Two *Sarcocystis* spp. with microscopic cysts (*S. tenella* and *S. arieticanis*) and two with macroscopic cysts (*S. gigantea* and *S. medusiformis*) infect sheep according to Dubey et al. (1989). Differentiation between the microscopic species was based on the morphological criteria described by Levine (1985) and Dubey et al. (1989). The present study revealed that the rate of infection in

heart muscles of sheep with microscopic *S. arieticanis* was 47.27%, comparing with results of El-Morsey et al. (2019) in Egypt that was 38.8% and Al Quraishy et al. (2014) that was, 40-53%. in different localities of K.S.A., and less than in Kanning City, China (91.9%) as reported by Hu et al. (2017). According to the available literatures, results of this study referred to the rate of infection according to gender and age that was not reported in all the previous studies. The ultrastructure of the cyst wall in the present specimens was "type 7" according to the classification of cyst walls reported by Dubey et al. (2016).

In this study, *Sarcocystis* sp. was microscopic and septated and had a thin cyst wall with horizontally branched finger-like protrusions that appeared parallel to the surface; they contained vesicles at their base, and the cyst wall protrusions did not have dome-shaped bases. This appearance is similar to that seen in the electron micrographs of Heydorn and Melhorn (1987); Odening et al. (1995); Hazirolu et al. (2003) and Al Quraishy et al. (2014). There were some morphological differences (e.g., size and shape of protrusions) between the present specimens and those from previous reports that described *S. arieticanis* from organs other than heart muscle, including the esophagus, diaphragm and tongue muscles of sheep (Dubey et al., 1988; Bittencourt et al., 2016; Hu et al., 2017; El Morsey et al., 2019). Those reports described the

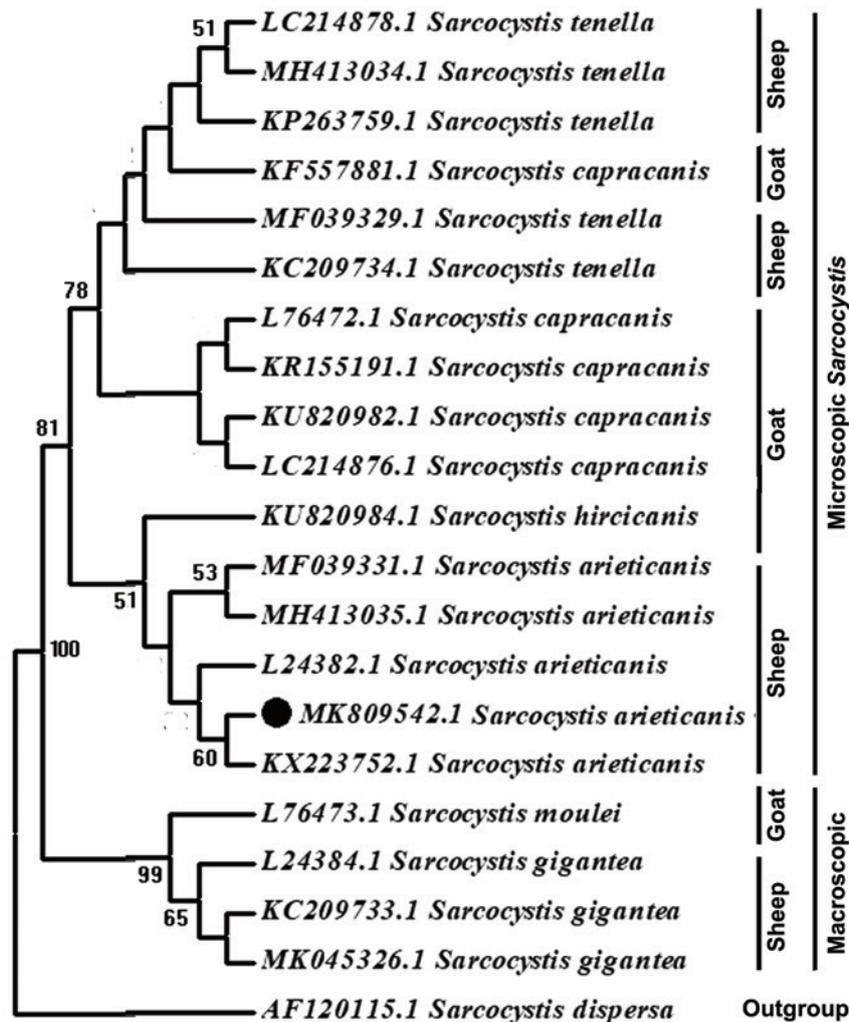


Fig. 7. Phylogenetic tree for *Sarcocystis* taxa known to infect sheep and goats only based on 18S rRNA sequences and inferred by the maximum likelihood (ML) method. The analysis involved 20 nucleotide sequences whose GenBank accession numbers are shown behind the taxon names. Numbers beside each branch show bootstrap values; values below 50% are not shown.

presence of a dome-shaped base in the cyst wall protrusions that was absent in the present specimens. This morphological characteristic may be related to the nature of the host muscles and cyst age; protrusions of the cyst walls may be folded in older cysts (Hazirolu, 2003), the morphology may change with age (Dubey et al., 1989) and the cyst walls may differ depending on the muscle tissues in which they are developing (Hazirolu et al., 2003). The ultrastructure of the present cysts was similar to that of *Sarcocystis* spp. in different Caprinae; for example, *S. arieticanis* in wild sheep (*O. musimon*), *S. hircicanis* in goats (Hu et al., 2016), *S. hircicanis/arieticanis*-like in blue sheep (*Pseudois nayaur*), Japanese serow (*Capricornis crispus*), and musk ox (*Ovibos moschatus*) (Odening et al., 1996). The cyst wall in specimens from the current study was thinner and differed from that in *S. capracanis* by having a thick- radially striated wall with finger-like projections (Hu et al., 2016; Morsy et al., 2011). The present *Sarcocystis* sp. differed from microscopic *S. tenella* from the esophagus and heart muscles of sheep, with the latter having palisade-like protrusions and unusual plaque structures in the tips of the villi (Hu et al., 2017; Hussein et al., 2018); it also differs from the microscopic thick-walled sarcocysts (*S. capracanis*) in goats (Mahran, 2009).

The phylogenetic tree (Fig. 7) shows that different species of sheep *Sarcocystis* did not form a single cluster and were not necessarily clustered in closely related clades; the same was observed for the different species of goat *Sarcocystis*. In fact, the sheep *Sarcocystis* (*S. arieticanis*, *S. tenella*, and *S. gigantea*) aggregated with the goat *Sarcocystis* (*S. hircicanis*, *S. capracanis*, and *S. moulei* respectively) forming three well-supported clades: *S. capracanis/S. tenella*, *S. arieticanis/S. hircicanis*, and *S. gigantea/S. moulei*. Furthermore, *S. gigantea/S. moulei* clustered at a distance from *S. arieticanis*, *S. tenella*, *S. hircicanis*, and *S. capracanis* in a separate clade. Morphologically, both *S. gigantea* and *S. moulei* were found to belonging to the macroscopic *Sarcocystis*, whereas *S. hircicanis*, *S. capracanis*, *S. arieticanis*, and *S. tenella* were seen to belong to the microscopic *Sarcocystis* (Dubey et al., 2016). Thus, the separation of *S. gigantea* and *S. moulei* from the *S. capracanis/S. tenella* and *S. arieticanis/S. hircicanis* clades can be attributed to their microscopic cyst size, inferring that cyst size is a strong differentiating morphological feature that should be considered first when classifying *Sarcocystis* spp.

In both phylogenetic trees *S. arieticanis* and *S. hircicanis* resolved as very closely related (Figs. 6 and 7). Both *S. arieticanis* and *S. hircicanis* are extremely similar, with no critical feature observed for distinguishing between them; they may be distinguished only by intermediate host specificity (Hu et al., 2016) as shown experimentally by unsuccessful transmission experiments with *S. arieticanis* in goats (Heydorn, 1985; Dubey et al., 1989) and with *S. capracanis* and *S. hircicanis* in sheep (Balbo et al., 1988; Dubey et al., 1989). Furthermore, sheep and goats (both of which have the same habitat, food type, and feeding manner) have been reported as harboring both *Sarcocystis* spp. (Dubey and Rosenthal, 2013; Formisano et al., 2013; Hu et al., 2016). Accordingly, the lack of distinctive characteristics for distinguishing between *S. arieticanis* and *S. hircicanis* as well as their very close phylogenetic relationship (and allied to their hosts' shared food type and feeding habitats), it is not satisfactory to recognize *S. hircicanis* as a distinct species. *S. hircicanis* is highly likely just a synonym for *S. arieticanis*, through additional molecular characterization and further detailed morphological studies are recommended.

Conclusion

To the author's best knowledge, the current investigation is the first to demonstrate the presence of the microscopic *Sarcocystis arieticanis* in heart muscles of domestic

sheep slaughtered for human consumption in Upper Egypt, with evidence from ultrastructural and molecular identification of isolated sarcocysts and description of the different developmental stages of bradyzoites using TEM.

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Conflict of interest

The author declares no conflicts of interest associated with this manuscript.

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