

MOLECULAR CHARACTERIZATION OF SARCOCYSTIS FUSIFORMIS AND SARCOCYSTIS BUFFALONIS INFECTING WATER BUFFALOES (BUBALUS BUBALIS) FROM EGYPT

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ABSTRACT

Parasites of the genus *Sarcocystis* have the ability to infect wide range of animals, birds and reptiles. Water buffaloes are intermediate hosts for four *Sarcocystis* species; *S. fusiformis*, *S. buffalonis*, *S. levinei* and *S. dubeyi*. There was no previous molecular identification of *Sarcocystis* spp. parasitizing water buffaloes from Egypt. The current study was established to screen infection rates and apply genetic identification of the macroscopic *Sarcocystis* spp. infecting Egyptian water buffalos in El-Gharbia province, Egypt. *Sarcocysts* of both *S. fusiformis* and *S. buffalonis* were grossly examined through meat inspection in slaughterhouse, followed by histological identification of the detected sarcocysts. Finally, molecular identification using PCR, sequencing and phylogenetic analysis of the 18S rRNA gene was conducted. Comparison of the obtained sequences of the local tested isolates of both *S. fusiformis* and *S. buffalonis* with those previously reported for different *Sarcocystis* species those were recorded in Genbank, revealed that all the tested specimens represented both *S. fusiformis* and *S. buffalonis*. Moreover, sequence alignments of the partial 18S rRNA gene ascertained the presence of genetic variations revealing the heterogenic nature of the gene among different strains of the same *Sarcocystis* species in relation to the geographic distribution.

Keywords: Sarcocyst, 18S rRNA, Phylogeny, Water Buffalo, Egypt

1. INTRODUCTION

Sarcocystis is a large genus of intracellular, cyst-forming coccidian parasites belonging to the protozoa (Sporozoa: Apicomplexa) with an obligate two host life cycle between predators as final hosts and their prey animals as intermediate hosts. *Sarcocystis* spp.

Are highly prevalent in livestock and are considered to be host specific (Dubey *et al.*, 1989).

Few *Sarcocystis* species are pathogenic to animals with a great economic impact as they cause reduced weight gain, poor feeding efficiency, anorexia, fever, anemia, muscle weakness, reduced milk yield, abortion and mortality of intermediate hosts such as *S. cruzi* in cattle, *S. tenella* in

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sheep, *S. capricanis* in goats and *S. miescheriana* in swine (Dubey *et al.*, 1989; El-Morsefy, 2010; El-Morsefy *et al.*, 2014). Macroscopic visible cysts, like *S. fusiformis*, *S. buffalonis* and *S. gigantea*, also may render meat unaesthetic and thus lead to condemnation of infected carcasses (Dubey *et al.*, 1989; Yang *et al.*, 2000; Hilali *et al.*, 2011). To date, four characterized species of *Sarcocystis* have been reported and described in the water buffalo as an intermediate host. *S. fusiformis* and *Sarcocystis buffalonis* form macroscopic sarcocysts with cats as the definitive host, while *Sarcocystis levinei* and *Sarcocystis dubeyi* form microscopic sarcocysts. Despite dogs are known to be the definitive hosts for *S. levinei*, the definitive host (s) for *S. dubeyi* has not yet been identified (Huong, 1999a; 1999b; Huong and Uggla, 1999; Huong *et al.*, 1997; Hilali *et al.*, 2011).

The present work aimed to conduct prevalence and molecular identification of the macroscopic *Sarcocystis* species infecting Egyptian water buffaloes in El-Gharbia province, Egypt, using gross identification by meat inspection, histological examination and molecular identification of the revealed *Sarcocystis* species using PCR, sequencing and phylogenetic analysis of (the small ribosomal subunit) 18S rRNA gene.

2. MATERIALS AND METHODS

2.1. Collection of Samples

Specimens were obtained from 528 water buffaloes slaughtered at the municipal slaughterhouse in El-Mahalla city, El-Gharbia province (coordinates: 30.881°N 31.06°E), Egypt during the period from December 2011 to November 2012. According to age, buffaloes were categorized into 2 groups, the first from 2-3 years and the second included animals of more than 5 years. Age of examined animals was determined based on the visual examination of both horns and teeth.

2.2. Gross Examination and Histopathology

Oesophageal muscles were examined by the naked eye to detect macroscopic sarcocysts. Macroscopic visible sarcocysts were carefully isolated from muscle samples and fixed in 10% formalin and routinely processed for histopathologic examination, sectioned at 5 µm and examined at magnifications of 40×, 100×, 400× and 1000×.

2.3. Molecular Identification

Prior to molecular confirmation, species identification was done on freshly collected muscle samples containing cysts of both species, using light microscopy according to (Dubey *et al.*, 1989; Odening *et al.*, 1995; 1996). Fresh sarcocysts of the two identified species were isolated from

muscle tissues with sterile brand needles for each cyst, washed 3-4 times with 0.9% NaCl as previously described according to (Yang *et al.*, 2001b). Five macroscopic sarcocysts were obtained from each of the identified *Sarcocystis* species. Samples were incubated into phosphate buffer saline (pH 7.2) and transferred as soon as possible to the Biotechnology Center, Faculty of Veterinary Medicine, Cairo University for further processing.

2.4. DNA Extraction

DNA was extracted from sarcocysts obtained from both isolates identified as *S. fusiformis* and *S. buffalonis* using Qiagen DNeasy Blood and Tissue kit[®] according to manufacturer protocol.

2.5. PCR Amplification

PCR reactions were applied for five macrocysts from both isolates of *S. fusiformis* and *S. buffalonis* using the primer set: Forward (5'-CGCCCTTTTAGTGAGGGTGT3') and reverse (5'-TACGAATGCCCAACTGTC 3') those were designed according to (18S rRNA sequence of *S. fusiformis* accession number U03071), using the primer BLAST tool on the NCBI of the Genbank (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/primertool.cgi>), to target the 18S rRNA gene in a final reaction volume of 50 µL containing 1 µL DNA template (100 ng), 0.5 µL (50 pmol) of each primer, 2 µL dNTPs (Advanced Bio-enzymes, UK), 1 µL DNA polymerase (2 U) (Advanced Bio-enzymes, UK), 5 µL of 10× buffer (500 mM KCl, 100 mM Tris HCl pH 9.0, 1.5 mM MgCl₂) and 40 µL Distilled water. Initial denaturation at 94°C for 4 min, 40 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 90 sec and then final extension at 72°C for 1 min. The amplification reactions were carried out in PCR Thermocycler (PTC 100 MJR Research, USA). Two µL of bromophenol blue were added to the aliquots of PCR products and the corresponding amplicons were electrophoresed on 2.5% agarose gel, stained with ethidium bromide, examined using a UV transilluminator and photographed.

2.6. DNA Sequencing, Sequence Alignment and phylogenetic Analysis

PCR amplicons were purified and prepared for sequencing using Qiaquick gel extraction kits[®] according to manufacturer recommendations. The sequence of the clones was determined from forward direction (5'-3'). Multiple DNA sequence alignments were carried out for 2 PCR products, one from each species. The 18S rRNA gene fragments were sequenced using the same primers used in PCR. Samples were analyzed on an ABI prism

377 capillary DNA sequencer. The sequence alignments were extensively checked by eye to determine the percentages of similarities and discrepancies. Initially, comparison was done between the sequences of the local tested isolates of *S. fusiformis* and *S. buffalonis*, then between the sequence alignments of the two species with those of previously published species of *S. fusiformis* AF179627, AF179626, (Yang *et al.*, 2001b) and U03071, (Holmdahl *et al.*, 1994) and *S. buffalonis* AF 017121, (Holmdahl *et al.*, 1999) to determine the percentages of similarities and variability among them. To reconstruct the phylogenetic relationship, 421 bp of the 18S rRNA gene representing each of PCR products of the two isolates (accessions numbers JQ821390-JQ821391) were included in a comparative analysis with those previously published in the NCBI using BLAST (<http://blast.ncbi.nlm.nih.gov/>), on the basis of highly similar sequences available on Genbank data base and by means of maximum parsimony trees reconstructed from Kimura 2-parameter distances from 1,000 bootstrap replicates of multiple sequence alignments created using CLUSTALW, as implemented by MEGA version 4 (Tamura *et al.*, 2007) The *Neospora caninum* (GQ 899206) was employed as outgroup.

3. RESULTS

3.1. Prevalence study

The present investigation revealed higher infection rates with both *Sarcocystis* species in old-aged animals (5 years and above), than those occurred in younger animals (2-3 years). Among old-aged animals, 68.2% (253/371) and 13.2% (49/371) were found to be infected with *S. fusiformis* and *S. buffalonis*, respectively. Infection rates in younger animals were 17.2% (27/157) and 10.2% (16/157). Mixed infections with both species were found in 83.8% (311/371) and 29.9% (47/157) for *S. fusiformis* and *S. buffalonis*, respectively (Table 1).

3.2. Morphologic Identification

S. fusiformis cysts were macroscopic, opaque white in color, spindle shaped, measured 0.5-3.0×0.3-0.7 cm and located sometimes under the serosal sheet or deeply situated within the esophageal musculature (Fig.1).

Double opposing arrow heads refer to the true cyst wall, single arrows refer to the nuclei of the muscle fiber layer adherent to the cyst wall, S; septa, M: peripheral metrocytes, B; bradyzoites (located toward the center of the cyst). Bar = 10 microns (H and Estain).

Histologically; the cyst was surrounded by smooth, thin cyst wall (1-3 μm). Fine Septa (S) originated from the inner layer dividing the cavity of the cyst into several compartments of different sizes, those were packed with

faintly-stained Metrocytes (M) in the periphery and Bradyzoites (B) toward the center (Fig. 2).

On the other hand, *S. buffalonis* cysts were visible to the naked eye as white threads running parallel to the longitudinal axis of the muscle fibers; often immediately under the serosal sheet of the esophageal muscles. They were 1-3×0.1-0.5 mm (n = 50). In few cases, cysts of *S. buffalonis* might appear twisted due to the postmortum tension of the underlying muscle fibers, (Fig. 3) and surrounded by a striated thick cyst wall measured 4-6 μm (n = 25) (Fig. 4). The internal structure was similar to that of *S. fusiformis*.

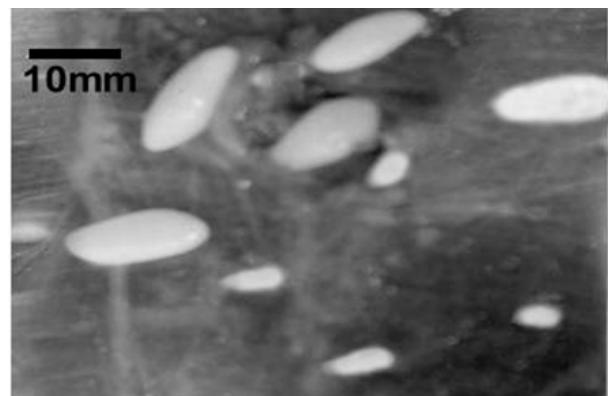


Fig. 1. Macroscopic *S. fusiformis* cysts in the esophageal muscle of water buffalo. Bar = 10 mm

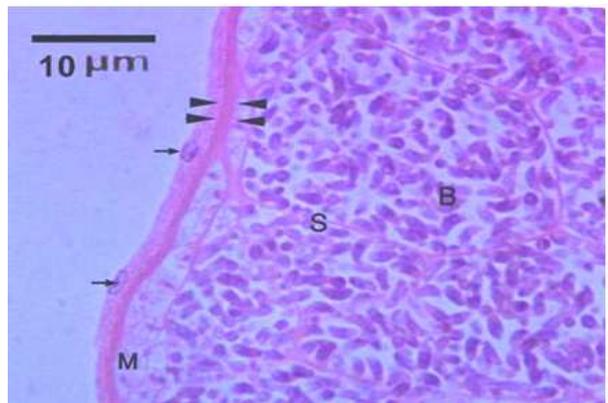


Fig. 2. Histological section of a mature *S. fusiformis* cyst showing the basic elements of the sarcocyst: Double opposing arrow heads refer to the true cyst wall, single arrows refer to the nuclei of the muscle fiber layer adherent to the cyst wall, S; septa, M: Peripheral metrocytes, B; bradyzoites (located toward the center of the cyst). Bar = 10 microns. (H and Estain)

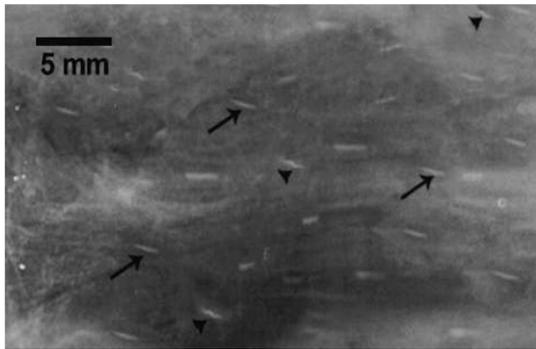


Fig. 3. *S. buffalonis* in the esophageal muscle of water buffalo showing thread like cysts in the oesophagus of water buffalo (arrows), some times appear twisted due to post mortum tension of the muscles (arrow heads). Bar = 5 mm

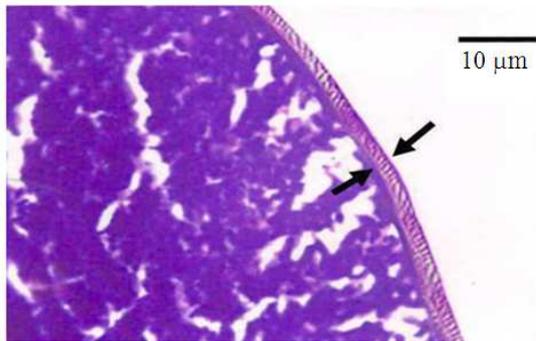


Fig. 4. *S. buffalonis*: Histological section of the cyst from the esophageal muscle of water buffalo, showing cross section of the cyst showing striated (palisade like) thick cyst wall (opposing arrows). (H&E) stain. Bar = 10 microns



Fig. 5. Electrophoresis of the 18S rRNA gene used for PCR assay for the local tested isolates of *S. fusiformis*. Lane M 100-bp ladder = DNA marker. Lanes 1-5 represent the positive diagnostic bands of the tested specimens

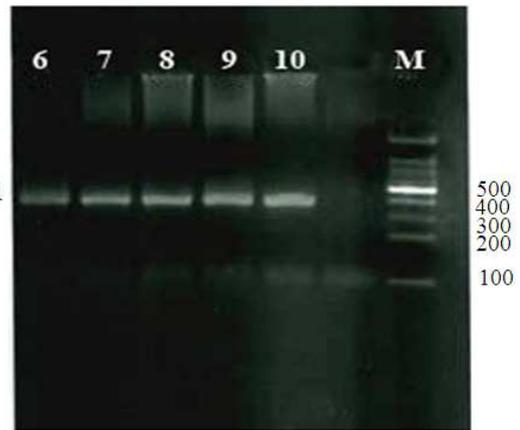


Fig. 6. Electrophoresis of the 18S rRNA gene used for PCR assays for the local tested isolates from *S. buffalonis*. Lane M 100-bp ladder = DNA. Lanes 6-10 represent the positive diagnostic bands of the tested specimens

3.3. Molecular Identification

The revealed diagnostic DNA bands representing the amplified target gene 18S rRNA appeared at 490 bp and 421 bp for *S. fusiformis* and *S. buffalonis*, respectively (**Fig. 5 and 6**).

The assembling of DNA sequences in both directions using forward and reverse primers yielded a fragment containing 421 consensus nucleotides for both sequences of the two isolates, *S. fusiformis* and *S. buffalonis*. Depending up on the BLAST comparative analysis, the partial 18S rRNA gene sequences of our local tested isolates of *S. fusiformis* and *S. buffalonis* showed (9%) dissimilarity, (**Fig. 7**). The similarity percent of Egyptian strain of *S. fusiformis* with the published data of *S. fusiformis* under accession number U03071; (Holmdahl *et al.*, 1994) was (99%). On the other hand, the similarity between the present *S. fusiformis* and published data of *S. fusiformis* (accession numbers AF176926 and AF176927; (Yang *et al.*, 2001b) was (97%). Whereas, sequence dissimilarities between our isolate of *S. fusiformis* were (7%) and (8%) with *S. buffalonis* (accession number AF017121) and *S. hirsuta* (accession number AF017122), respectively.

Meanwhile, the sequence identity between the current isolate of *S. buffalonis* and published data of *S. buffalonis* (accession number AF017121) and *S. hirsuta* (accession number AF017122) were (98%) and (97%) respectively (**Fig. 8**).

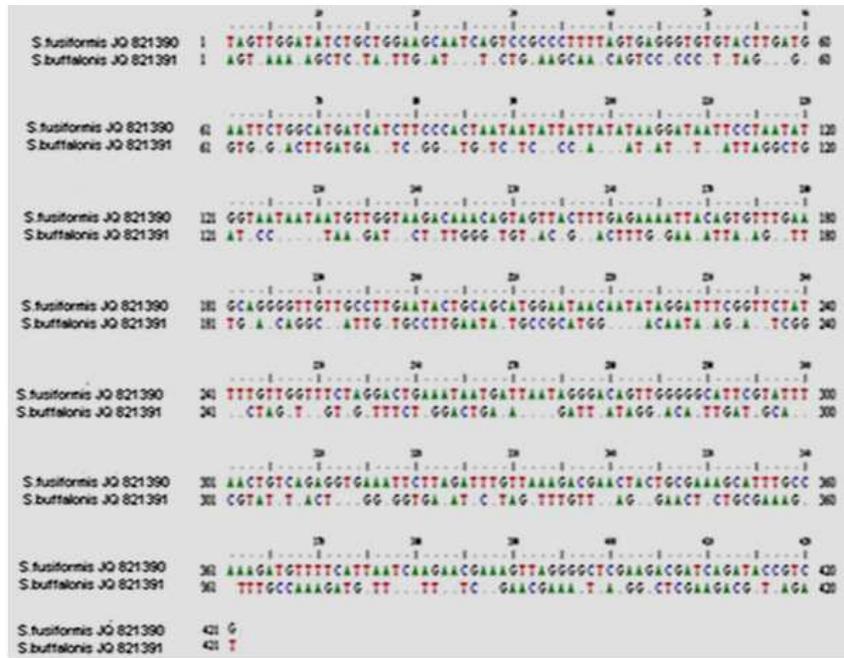


Fig. 7. Sequence alignments of the 18S rRNA gene consisting of 421 nucleotides for each of the local tested isolates of *S. fusiformis* (GenBank accession number JQ821390) and *S. buffalonis* (GenBank accession number JQ821391) from Egyptian water buffaloes

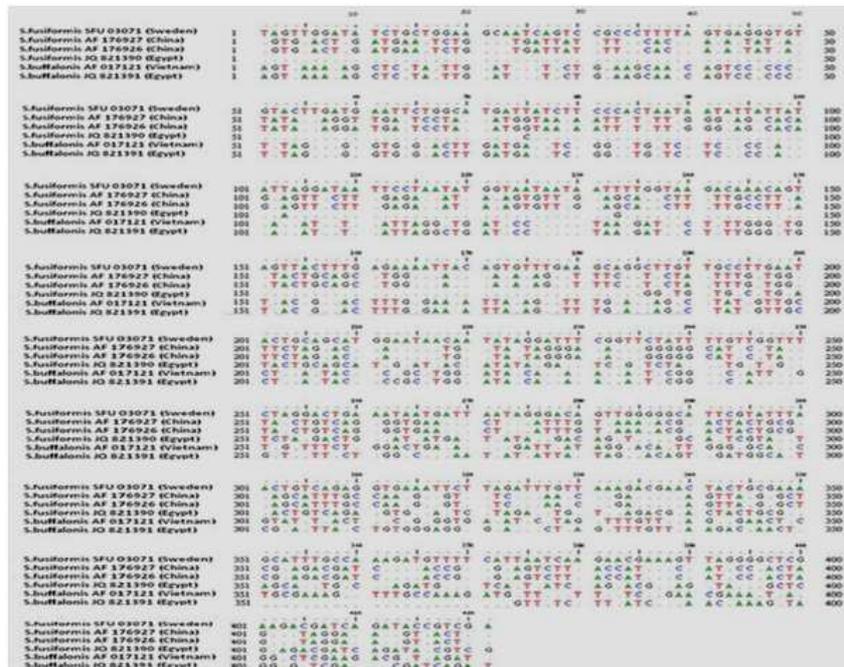


Fig. 8. Sequence alignment of 18S rRNA gene for local tested isolates of both *S. fusiformis* and *S. buffalonis* compared with the published sequences of the same gene. The alignment was performed using MEGA version 4

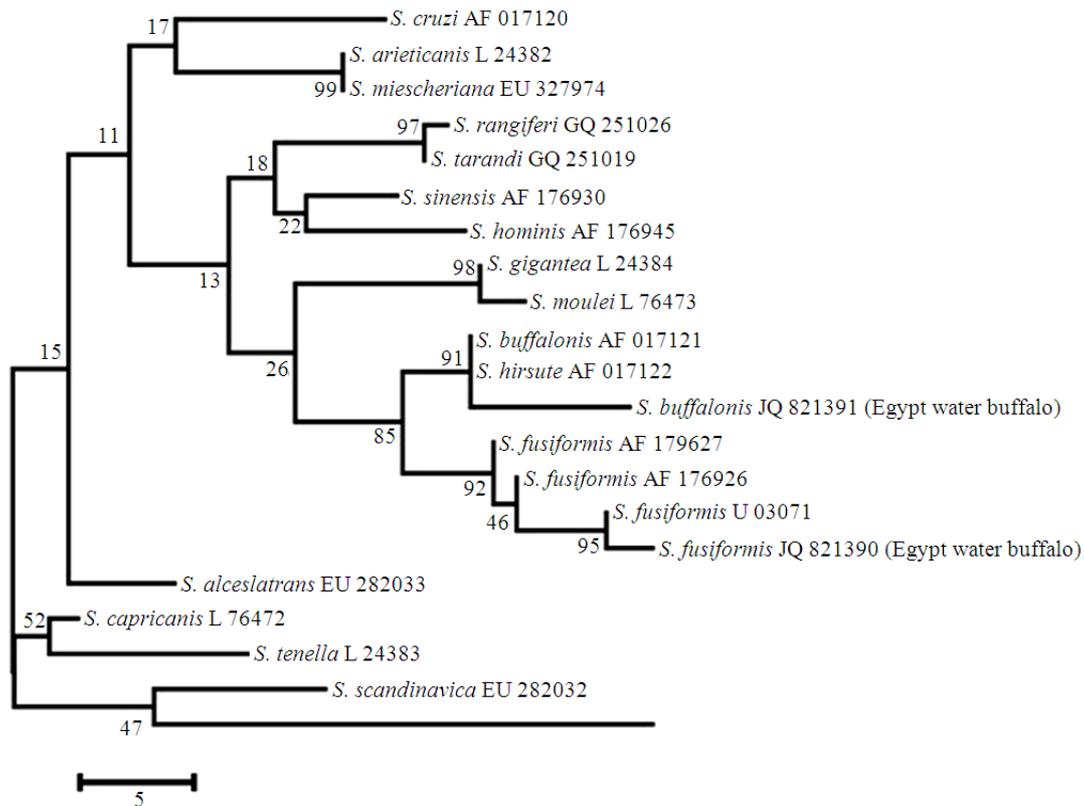


Fig. 9. Cladogram (phylogenetic tree) of *Sarcocystis* species based on the 18S rRNA gene sequence constructed according to maximum parsimony method. The branch lengths are proportional to the amounts of evolutionary changes. The phylogenetic tree indicates

The phylogenetic analysis of the 18S rRNA sequences using *N. caninum* (GQ899206) as outgroup, showed that the Egyptian isolate of *S. fusiformis* placed in a group with the three previously reported *S. fusiformis* strains (accession numbers U03071, AF176926 and AF176927). Furthermore, the cladogram revealed that this group was more associated with the newly identified *S. buffalonis* isolates from the Egyptian water buffaloes (accession number JQ821391) together with *S. buffalonis* (AF017121) and *S. hirsuta* (AF017122) (Fig. 9).

4. DISCUSSION

Sarcocystosis is common within a wide host range of vertebrates as well as humans (Abdel-Ghaffar *et al.*, 1978; 1990; Abdel-Ghaffar and Al-Johany, 2002).

Few decades ago, the prevalence of *Sarcocystis* species in Egyptian water buffaloes (*Bubalus bubalis*) has been reported from different regions in Egypt, with few

investigations studied detailed identification of the revealed *Sarcocystis* species. (Abdel-Ghaffar *et al.*, 1978) examined the oesophagi of 20 Egyptian water buffaloes aged 7-10 years and found that all of them were infected with *S. fusiformis*. In addition, (Khalifa *et al.*, 2008) examined specimens from tongue, heart, esophagus and skeletal muscles of 100 water buffaloes slaughtered in Sohag province, Egypt, revealing an infection rate of 28% with only *S. fusiformis*. El-Dakhly *et al.* (2011) examined 379 water buffaloes from the main slaughterhouses of Beni-Suef province, Egypt and found that 299 were infected with overall prevalence of 78.9%. Based on age, the examined animals were categorized into 3 groups. Infection rates with both macroscopic, *S. fusiformis* and microscopic, *S. levinei*, were 74.5, 82.3 and 81.2% in animals aged 1.5-2 years, adult females aged 2-5 years and females aged over 5 years, respectively. Moreover, (Hilali *et al.*, 2011) recorded, for the first time, the infection of water buffaloes in Egypt with the microscopic *S. dubeyi* species with a prevalence rate of 30%.

Table 1. Prevalence rates with *S. fusiformis* and *S. buffalonis* among the two groups of Egyptian water buffaloes (*Bubalus bubalis*) relative to age during the period extending from December 2011 to November 2012

Age groups	Total numbers of examined animals	Animals infected with <i>S. fusiformis</i>		Animals infected with <i>S. buffalonis</i>		Animals with mixed infection	
		No	%	No	%	N	%
2-3 years	157	27	17.2	16	10.2	47	30
5 years and above	371	253	68.2	49	13.2	311	84

In the current study, infection rates with *S. fusiformis* were 68.2% in old-aged water buffaloes (5 years and above) and 17.2% in younger age group (2-3 years). The rate of infection with *S. buffalonis* in old-aged animals was 13.2%, while it was 10.2% in younger age group. Mixed infection was observed in 83.8% of the old-aged group, while it was 29.9% in younger animals (**Table 1**).

The present investigation revealed the occurrence of *S. fusiformis* and *S. buffalonis* in water buffaloes in Egypt with the first molecular characterization of them in the country. However, both species were reported from water buffaloes worldwide including China (Li *et al.*, 2002; Yang *et al.*, 2001b). Vietnam (Jehle *et al.*, 2009) and Iran (Oryan *et al.*, 2011). Huong (1999a; 1999b) reported four species *Sarcocystis*, *S. levinei*, *S. dubeyi*, *S. buffalonis* and *S. fusiformis* in Vietnam. Moreover, (Claveria and Cruz, 2000) showed *S. levinei* in Philippines' water buffaloes.

Depending on the main fact that the morphometric characteristics of the cyst wall are the basic criteria for identification of different *Sarcocystis* species within the same intermediate host (Dubey *et al.*, 1989; Xiang *et al.*, 2010; El-Morsey *et al.*, 2014). Moreover, Xiang *et al.* (2010) stated that light microscopy might be used to demonstrate the occurrence of sarcocysts in mammalian tissues and could be employed to discriminate among some parasite species based on the cyst wall structure as well as the size of the sarcocysts. Initially, the present work reported that water buffaloes in Egypt were infected by another macroscopic species, namely *S. buffalonis*, which was first previously recorded in Vietnam by (Huong *et al.*, 1997). In the current investigation, cysts of *S. buffalonis* measured 1-3×0.1-0.5 mm and were comparatively smaller than those previously described by (Huong *et al.*, 1997) (1-8×0.1-0.5 mm) in Vietnam. Furthermore, *S. buffalonis* cysts, described herein and were surrounded by striated thick cyst wall measured from 4-6 µm, while (Huong *et al.*, 1997) reported that the cyst wall measured 3-7.7 µm thick.

S. buffalonis sarcocysts measured 1-3 mm long and appeared thread-like under the perimysial connective tissue along the longitudinal axis of the muscle fibers.

Some specimens appeared twisting as a result of the postmortem contraction of the muscle fibers.

With light microscopy, the cyst wall of *S. buffalonis* appeared thick, measuring 4-6 µm and characterized by the presence of striations (palisade-like). On the other hand, *S. fusiformis* cysts had thin and smooth wall that measured 1-3 µm. *S. buffalonis* cysts were only detected in the oesophageal muscles (skeletal muscles were not included). Huong *et al.* (1997) found them in skeletal muscles, mainly abdominal muscles (49%), cervical muscles (44%), esophagus (27%) and tongue (25%) with the heart was free. Huong (1999a) reported that *S. buffalonis* were present in cervical muscles (14.3%), abdominal muscles (16%), tongue (8%) and no infected cases in the heart. On the other hand, (Oryan *et al.*, 2010) conducted the distribution pattern of *Sarcocystis* species in slaughtered water buffaloes (*Bubalus bubalis*) in Khuzestan, Iran by macroscopic and histological examination. They investigated esophagus, heart, diaphragm, tongue, masseter and thigh muscles. Among 100 examined water buffaloes, three (3%) cases had infection with macroscopic *Sarcocystis* in the esophagus and thigh muscles, while 83% (83/100) revealed microscopic cysts. The highest prevalence of microscopic cysts was found in masseter muscle (57.1%), followed by tongue, diaphragm, esophagus, heart and finally, thigh muscles (30.0%). There was no significant difference between males (83.6%) and females (82.0%). They relayed on the size of the sarcocysts, wall thickness and location of cysts, as basis for species identification. Macroscopic, *S. buffalonis* and microscopic forms, *S. levinei* and *S. dubeyi*, were found in examined muscles of water buffaloes, while *S. fusiformis* sarcocysts could not be detected in the internal organs of the examined water buffaloes.

Recently, the 18S rRNA genes have been extensively used as suitable targets for the accurate identification of the closely related species of *Sarcocystis* as well as phylogenetic analyses (Holmdahl *et al.*, 1999; Li *et al.*, 2002; Dahlgren and Gjerde, 2007; Jehle *et al.*, 2009). The conserved regions of 18S rRNA gene help in designing primers that can be used to amplify the same

gene in related species. Its mosaic structure allows flexibility in experimental designs for various phylogenetic studies (Olsen and Woese, 1993). Furthermore, within *Sarcocystis* species, the sequences of this gene have variable genotypic behaviors (Holmdahl *et al.*, 1999). In addition, (Holmdahl *et al.*, 1999) found that the 18S rRNA gene showed a high congruence of identity values within species, so it is a valuable to identify *Sarcocystis* species based on analysis of this gene.

The results of phylogenetic analysis revealed that there was a far relationship between the local tested isolates of both *S. fusiformis* and *S. buffalonis* according to achieved sequences of 18S rRNA gene and the phylogenetic tree analysis (Fig. 9). Meanwhile, the cladogram indicated that our local tested samples of *S. buffalonis* (accession number JQ821391) was closely related to *S. buffalonis*, accession number AF017121 (Holmdahl *et al.*, 1999) and this was reflected by the high nucleotide homology (98%) between them. On the contrary, the relationship with the tested isolate of *S. fusiformis* (accession number JQ821390) was highly different and represented by (9%) nucleotide dissimilarity, suggesting a distinct genetic difference between the tested isolates of *S. buffalonis* and *S. fusiformis* investigated in the current study. However, at the same time, all these *Sarcocystis* species are related to each other as they utilize water buffaloes and cats as intermediate and final hosts, respectively. Holmdahl *et al.* (1999) stated that most of the *Sarcocystis* species clearly differed from one another in more than 100 nucleotides positions with the exception for *S. buffalonis* and *S. hirsuta* (13 positional differences). Moreover, the genotypic similarity of these species was also reflected in their close phenotypic resemblance (both species are macroscopic, thread-like and have cats as final hosts despite *S. buffalonis* is a parasite of water buffalo and *S. hirsuta* is a parasite of cattle). Yang *et al.* (2001a) mentioned in their analysis that the minimum relative sequence diversity between *S. fusiformis* and *S. hirsuta* was 1.7%. Significant macroscopic, histological differences as well as data of sequence and phylogenetic analyses suggest a high genetic variability between both local tested isolates of *S. fusiformis* and *S. buffalonis*.

In the present study, phylogenetic analyses revealed that sequences of the local tested isolates of *S. fusiformis*, *S. buffalonis*, *S. buffalonis* (AF017121) and *S. hirsuta* (AF017122) shared the same definitive host (cat) and formed one cluster.

The 18S rRNA gene is characterized by the presences of various loci of polymorphism between

the different species of *Sarcocystis* and within same species (Holmdahl *et al.*, 1999; Oryan *et al.*, 2011) this was observed in the current study and indicated by differences in the nucleotide sequences from the same *Sarcocystis* sp. Accordingly, the present Egyptian *S. fusiformis* differed by (3%) and (1%) from those previously recorded from China and Sweden, respectively (Yang *et al.*, 2001b; Holmdahl *et al.*, 1994). Therefore, examination of more sarcocysts from other geographic locations and sequencing at more genetic loci (for example, 28S rRNA and ITS-1) may elucidate whether there are more differences within the same species. Another possibility of nucleotide variability within the same *Sarcocystis* species is that, water buffaloes in different geographic regions may harbor different strains of the parasite. Moreover, (Oryan *et al.*, 2011) revealed that the 18S rRNA gene possesses different genetic variants which may arise as a result of dissimilarities among the multiple copies of this gene that were amplified from different merozoites within the sarcocyst.

5. CONCLUSION

Higher infection rates with both macroscopic *S. fusiformis* and *S. buffalonis* spp. within older age water buffaloes were observed. This may be due to that the sarcocysts need longer time to appear macroscopically. Economic losses may occur as a result of condemnation of carcasses infected with macroscopic sarcocysts in addition to infection with some pathogenic species of the sarcocyst cause weight gain, poor feeding efficiency, anorexia, fever, anemia, muscle weakness, reduced milk yield, abortion and mortality of intermediate hosts. To the best knowledge of the authors, the present work included the first molecular identification of *S. fusiformis* in Egypt, together with the first record of infection of Egyptian water buffaloes with *S. buffalonis*, depending up on both the morphological characteristics of the two species and the molecular differences between the two isolates as deduced from the analyses of 18S rRNA gene sequences. However, further investigations on other genetic markers such as 28 S rRNA and ITS1 genes, are required for complete differentiation between currently detected *Sarcocystis* spp and the formerly identified species those were recorded from various geographic localities. Strict control measures should be applied on stray dogs and cats in developing countries especially Egypt, as they play serious roles in transmitting infection with different species of *Sarcocystis* to water buffaloes.

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