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PREVALENCE AND GENETIC CHARACTERIZATION OF *Sarcocystis fusiformis* IN WATER BUFFALOES (*Bubalus bubalis*) IN TWO NORTHERN PROVINCES OF EGYPT

Soad M. Menshawy[1], Bothaina H. Essa[2], Sabah I. Shaaban[3], Mohamed Hamada[4], Sahar F. Mahmoud[5], Mahmoud AbouLaila[1]*, Shimaa S. Sorour[6]

[1]Department of Parasitology, [2]Department of Animal Husbandry and Animal Wealth Development, [3]Department of Animal Hygiene and Zoonoses, [5]Department of Histology and Cytology, Faculty of Veterinary Medicine, Damanhour University, 22511 Damanhour, Elbehera, [4]Department of Food Hygiene and Control, Faculty of Veterinary Medicine, University of Sadat City, 32511 Sadat City, Menofia, [6]Department of Parasitology, Faculty of Veterinary Medicine, Kafrelsheikh University, 335116 Kafrelsheikh, Kafrelsheikh, Egypt

*Corresponding author, E-mail: hethet2004@yahoo.com

Abstract: Sarcocystosis considerably occurs in a wide host range including animals, reptiles, humans, and birds. This study was conducted to determine the prevalence of *Sarcocystis* spp. using abattoir inspection, genetic characterization, as well as histopathology in water buffaloes in two provinces, Elbehera and Kafrelsheikh, Egypt. Specimens were collected from esophagus, tongue, and masseters of 400 slaughtered buffaloes in Elbehera (*n*= 215) and Kafrelsheikh (*n*= 185). Samples were examined macroscopically and histopathologically. Furthermore, genetic characterization of *Sarcocystis* spp. was performed using the 18SrRNA gene-based PCR. The total prevalence was 71.0% (75.3% and 65.9% in Elbehera and Kafrelsheikh, respectively). Aged buffaloes had a higher prevalence than young ones. Females had a higher prevalence than males. The esophagus was the most infected organ. Molecular analysis revealed that the recovered species was *S. fusiformis*. This is the first genetic characterization of *S. fusiformis* in water buffaloes from Elbehera and Kafrelsheikh, Egypt. Higher prevalence proposed the potential role of cats in the transmission of *S. fusiformis*, which, in turn, requires strict hygienic measures to protect animals and humans from infection.

Key words: *Sarcocystis fusiformis*; prevalence; genetic characterization; Buffalo; Elbehera; Kafrelsheikh

Introduction

Sarcocystis is an intracellular cyst-forming protozoan parasite belonging to the phylum Apicomplexa. It is an obligatory heteroxenous parasite causing sarcocystosis. Carnivores are the final hosts harboring sexual stages, while herbivores are the intermediate hosts with asexual stages (1, 2). Hence, each host can be infected with multiple *Sarcocystis* species (1). Sarcocystosis is an important parasitic disease that has a global distribution in humans and various animal species (3). These food-borne zoonotic parasites infect humans through raw or insufficiently cooked infected meat consumption (3,4). Some *Sarcocystis* species are economically important due to their ability to induce anorexia, anemia, muscular weakness, gastrointestinal problems, fever, and decreased weight gain and milk production (5). A few species can induce abortion and mortality in intermediate hosts, such as *S. tenella* in sheep, *S. cruzi* in cattle, *S. miescheriana* in swine, and *S. capricanis* in goats (1). Macroscopic cysts like *S. buffalonis, S. fusiformis,* and *S. gigantea* make meat unsuitable for human consumption, resulting in the condemnation of infected carcasses (6). Water

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buffaloes are intermediate hosts for *Sarcocystis* species, including *S. fusiformis* and *S. buffalonis*, which form visible cysts, with cats as final hosts, and dogs serving as final hosts for *S. levinei*. *S. dubeyi*'s final host is unknown (7). Macroscopic sarcocysts, which almost always occur in skeletal or esophageal muscles, appear filamentous in *S. muris*, rice grain-like in *S. rileyi*, fusiform in *S. fusiformis*, globular in *S. gigantea* (1), and white thread-like in *S. buffalonis* (8).

Several investigations were conducted to measure the prevalence of *Sarcocystis* spp. infections with varying prevalence rates, including Northern Vietnam (9), Iran (10, 11), India (12), China (13), and Ethiopia (14). In Egypt, infection was detected in water buffalos from a few provinces such as Beni-Suef (2), El-Gharbia (8), Dakahlia (15), Menoufia (16), and Sharkia (17). The serological diagnosis was conducted using ELISA in water buffaloes from Iran (12). Molecular characterization is important for distinguishing the cyst of *Sarcocystis* species with similar morphology. Several prior investigations used the 18SrRNA gene as a target gene for *Sarcocystis* species because it has hypervariable areas that might give useful information for identifying and describing various species within the same genus (18). It was employed either alone or in combination with other molecular markers. Except for a few studies that utilized the Cox I gene alone (19) or the Cox I gene with another genetic marker (20). Some studies in Egypt used molecular diagnosis using the 18SrRNA gene for instance in Beni-Suef (2) and El-Gharbia (8). In addition, there was no epidemiological or molecular data on *Sarcocystis* infection in water buffaloes in Elbehera or Kafrelsheikh provinces. This study aimed to provide morphology, histopathology, epidemiology, and molecular characterization of *Sarcocystis* spp. in water buffaloes in Elbehera and Kafrelsheikh provinces, Egypt.

Materials and methods

Study design and samples

This study was assigned to detect the *S. fusiformis* in slaughtered water buffaloes from Elbehera and Kafrelsheikh governorates in northern Egypt. The study animals were designated using a simple random sampling method that took age and gender into account. The sample size required for this study was estimated consistent with the following formula $N= 1.96^2 [Pexp-(1-Pexp)]/d^2$ where N represents the required sample size, Pexp represents the expected prevalence, and d represents the desired absolute precision (21). To increase the degree of precision, an expected prevalence of 50% was utilized, and 384 sample sizes were calculated using a 5% absolute precision and a 95% confidence level. The esophagus, tongue, and masseter muscles were collected from 400 slaughtered buffaloes from Elbehera (n = 215) and Kafrelsheikh (n = 185) provinces in northern Egypt during the period from May 2020 to April 2021. The age of animals was determined by tooth examination (22). Also, sex was recorded. The esophagus, tongue, and masseter muscles were examined during routine meat inspection. Then samples were sent to the laboratory of Parasitology at the Faculty of Veterinary Medicine, Damanhour University, Egypt for morphological and molecular examination.

Gross examination

Muscle samples from the esophagus, tongue, and masseter muscles were grossly examined during normal meat inspection at abattoirs to detect macroscopic *Sarcocystis* cysts.

Histopathology

Tissue specimens from the esophagus infected with macroscopic cysts and noninfected normal esophagus were fixed in 10% neutral-buffered formalin for 2–5 days. Then it was processed for paraffin embedding and sectioned into 5-7 µm thick sections. The sections were stained with hematoxylin and eosin (H&E) and examined microscopically (23).

DNA extraction and PCR amplification

Cysts were separated from the surrounding muscles and genomic DNA was extracted independently from each of *Sarcocystis* cysts from the esophagus (five cysts from each province) using the Genomic DNA Extraction Kit (Thermo Fisher Scientific Inc., MA, USA) (24). The concentration of DNA was measured using a spectrophotometer (Thermo Fisher Scientific, USA). PCR reactions were applied using the primer set: forward (5´-GGCCCTTTTAGTGAGGGTGT-3´) and reverse (5´-TACGAATGCCCCCAACTGTC-3´) of the 18SrR-

NA gene of *S. fusiformis* targeting 270bp fragment (24). PCR reactions were performed in a final reaction volume of 25 µL using 2× PCR Master Mix Solution (i-Taq™) (iNtRON Biotechnology, Seoul, Korea). Negative control Double-distilled water was performed with all the reactions. The PCR reaction conditions were similar to previous research (24). Five µL of PCR products were tested by running on a 2.5% agarose gel (Sigma-Aldrich, USA), stained with ethidium bromide (Sigma-Aldrich, USA), using a 100-bp genetic marker (Sigma-Aldrich, USA), then visualized using a UV and photographed. PCR products were purified using MEGAquick-spin™ Plus Total Fragment DNA Purification Kit (iNtRON Biotechnology, Seoul, Korea) according to the recommendations of the manufacturer.

Sequencing analysis and phylogenic tree

Purified PCR products were submitted for sequencing at Animal Health Research Institute, Giza, Egypt. The Basic Local Alignment Search Tool (BLAST) was used to align and compare DNA sequences with the GenBank database. The sequences were submitted to GenBank. The Mega 5 software was used to generate the phylogenetic tree by the Neighbor-Joining method. Sequences from Elbehera (OP388862) and Kafrelsheikh (OP388863) and other sequences from GenBank were used, including *S. fusiformis* (KR186117, KR186119, KR186122, MF327256, MN986970, MN986971, KX574317, MF595830, MF595833, MF595837, MF595838, MF595839, and MF595840), *S. cafferi* (KJ778010, KJ778011, and KJ778019), *S. buffalonis* (KU247902, KU247908, KU247909, KU247910, KU247911, KU247912, KU247913, MF595842, MF595843, and MF595844), *S. hirsute* (AF017122, AF176940, AF176941, KC209741, KT901160, MN121568, MN121569, MN121570, and MN121571), and *Plasmodium Vivax* (AY598140) as out-group.

Statistical analysis

Locality, age, and sex variables were analyzed by the Chi-square ($x2$) test using SPSS statistical software (Version 22, SPSS Inc, Chicago, MI, USA) to evaluate the relationship between these variables and *S. fusiformis* infection. P value ≤ 0.05 was considered statistically significant.

Results

The infection rate of Sarcocystis cysts in water buffaloes

The total prevalence of *Sarcocystis* cysts among water buffaloes was 71.0%. The infection rate was 75.3% in the Elbehera western and 65.9% in the Kafrelsheikh middle delta, Egypt (Table 1). Locality significantly affected the infection (x^2 = 4.2699, P = 0.038) (Table 1). According to age, the prevalence was higher (75.1%) in aged animals > 3 years than (49.2%) in young animals < 3 years. Age significantly affected the infection (x^2 = 17.2494, P = 0.0001) (Table 1). Female buffaloes had a greater prevalence of 75.9% than males (30.2%). (Table 1). Sex significantly affected the infection of *Sarcocystis* (x^2 = 38.8894, P = 0.0001) (Table 1). The esophageal muscles were the most infected organ during meat inspection, with a prevalence of 96.12% (74.4% and 61.1%), followed by the tongue at 3.87% (0.9% and 4.8%), whereas the masseter muscles were uninfected in Elbehera and Kafrelsheikh, respectively.

Table 1: Risk factors affecting the prevalence of Sarcocystis fusiformis in water buffaloes in Elbehera and Kafrelsheikh, Egypt

Variable		No. examined	No. positive	%	x2	P Value
Locality	Elbehera	215	162	75.3	4.27	0.038*
	Kafrelsheikh	185	122	65.9		
Age	< 3 years	63	31	49.2	17.25	0.0001****
	≥ 3 years	337	253	75.1		
Sex	Male	43	13	30.2	38.89	0.0001****
	Female	357	271	75.9		

* Significant at $P \leq 0.05$

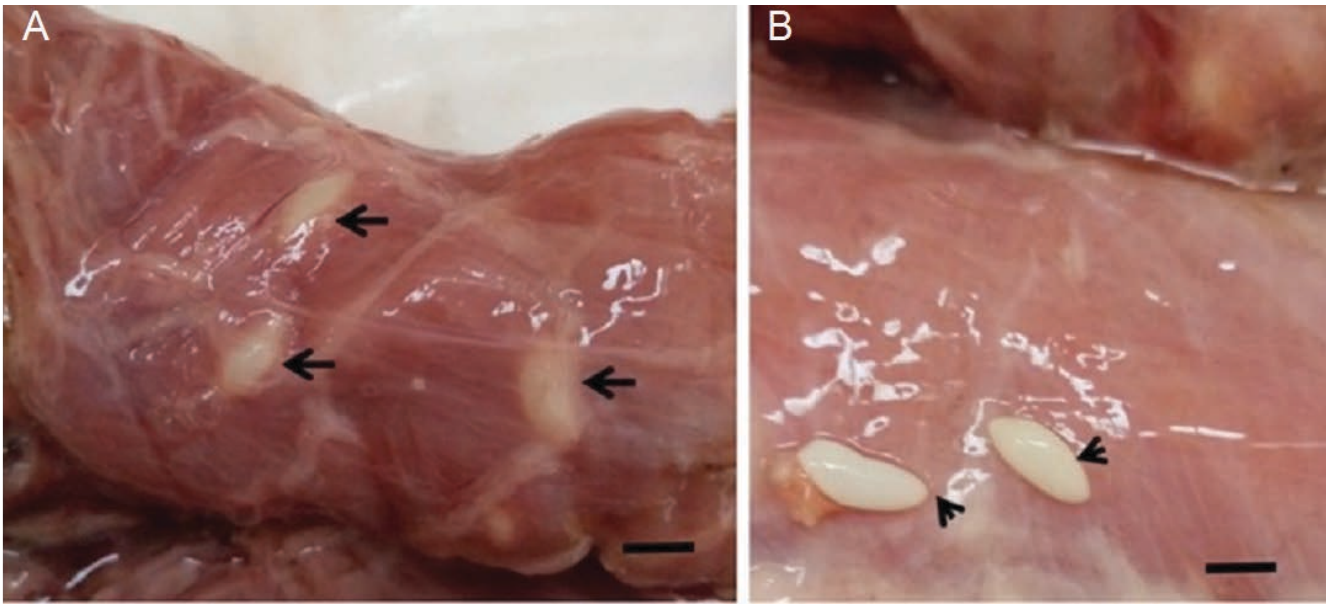

Figure 1: An esophagus of a water buffalo infected with macroscopic S. fusiformis cysts. A) Cysts are distributed beneath the serosal membrane (arrows) and B) the isolated macroscopic cysts from esophageal muscles (arrowheads), Scale bar = 1 cm

Gross examination

The species of *Sarcocystis* cyst was identified morphologically as *S. fusiformis* in esophageal and tongue muscles. The cyst was macroscopically fusiform in shape, with opaque white milky bodies in color, lying between muscle bundles in the same direction as the muscle mass and beneath the serosal membrane. It was found in different sizes, measuring about 0.5-3.0 cm (Figure 1).

Molecular characterization

The PCR primers revealed specific bands of 270bp for the 18SrRNA gene. *Sarcocystis* species sequences in buffalo from Elbehera (OP388862) and Kafrelsheikh (OP388863), Egypt, belonged to *S. fusiformis*. The 18SrRNA gene sequences of our tested isolates from Elbehera and Kafrelsheikh showed 97% similarity, according to BLAST analysis. The sequence identity between Elbehera isolate and published *S. fusiformis* (MF327256 and KR186119) was 99.5%. The Elbehera sequence shared 99% and 98% similarities with previously released *S. fusiformis* (KR186117 and KR186122), respectively. The Kafrelsheikh 18S rRNA sequence was found to have 97% sequence identity with *S. fusiformis* (MF327256 and KR186119). It exhibited sequence similarity of 96% with *S. fusiformis* (KR186117) and 95% with *S. fusiformis* (KR1861122). The cladogram showed that the Kafrelsheikh isolate was placed in a group with the *S. fusiformis* sequences in GenBank (KR186117, KR186122, MF327256, and KR186119), which are closely related to the Elbehera strain (Figure 3). The cladogram revealed that the sequences of *S. fusiformis* were genetically related to *S. cafferi*, followed by *S. buffalonis*, and then *S. hirsuta* (Figure 3).

Discussion

Sarcocystis species are intracellular zoonotic parasites that infect both animals and humans. We investigated them because they are common coccidian protozoan parasites of veterinary and economic importance. The total infection rate of *S. fusiformis* cyst among slaughtered water buffaloes was 71.0%. This high prevalence of *S. fusiformis* infection may be because of continuous exposure to infection via infected cats, the definitive host, shedding numerous sporocysts in their faces that contaminate the environment and serve as the primary source of infection (2). The locality had a significant effect on the infection which may be due to different husbandry management systems (25). Therefore, the difference in the prevalence may be due to geographic distribution (26) and the extent of exposure to infection. The prevalence was similar to 65% in the Philippines (27) and 68.2%,

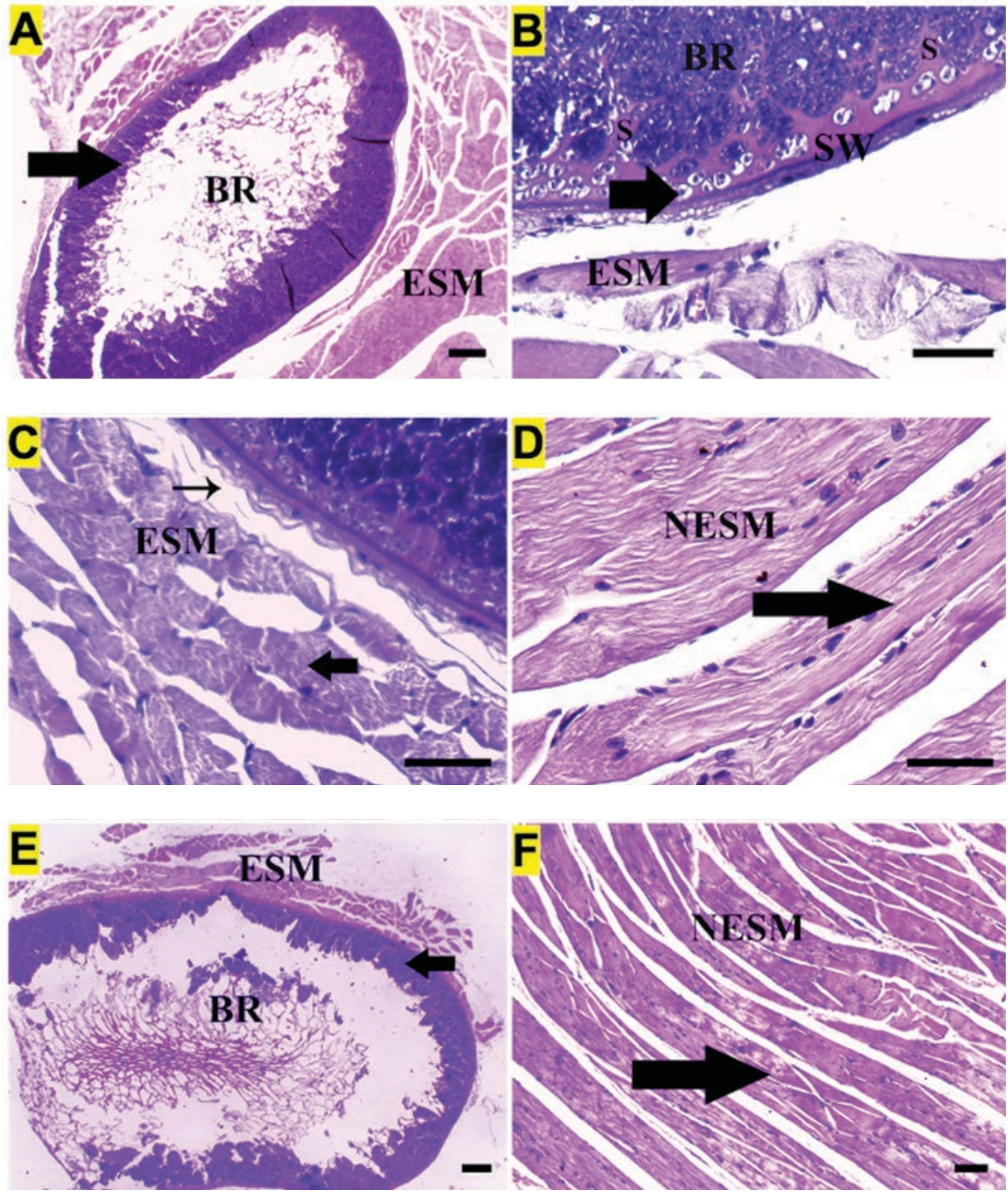

Figure 2: Photomicrograph of *S. fusiformis* from buffalo esophageal muscle in panels A, B, C, and E. Normal esophageal muscle without *Sarcocystis* in panels D and F. A) Esophageal skeletal muscle (ESM) containing *S. fusiformis* surrounded by a thin capsule (arrow) Scale bar = 200 µm. B) lightly stained metrocytes (arrow), bradyzoite (BR), scale bar = 50 µm. C) Muscle loss near the cyst (thin arrow), nucleus loss, and regular shape of striated cytoplasm (thick arrow), scale bar = 50 µm. D) Normal esophageal muscle (NEM) is shown, Scale bar= 50 µm. E) *S. fusiformis* cross-section (arrow) within detached esophageal skeletal muscle (ESM), scale bar= 200 µm. F) NEM with clear striation and arrangement (arrow), scale bar= 200 µm

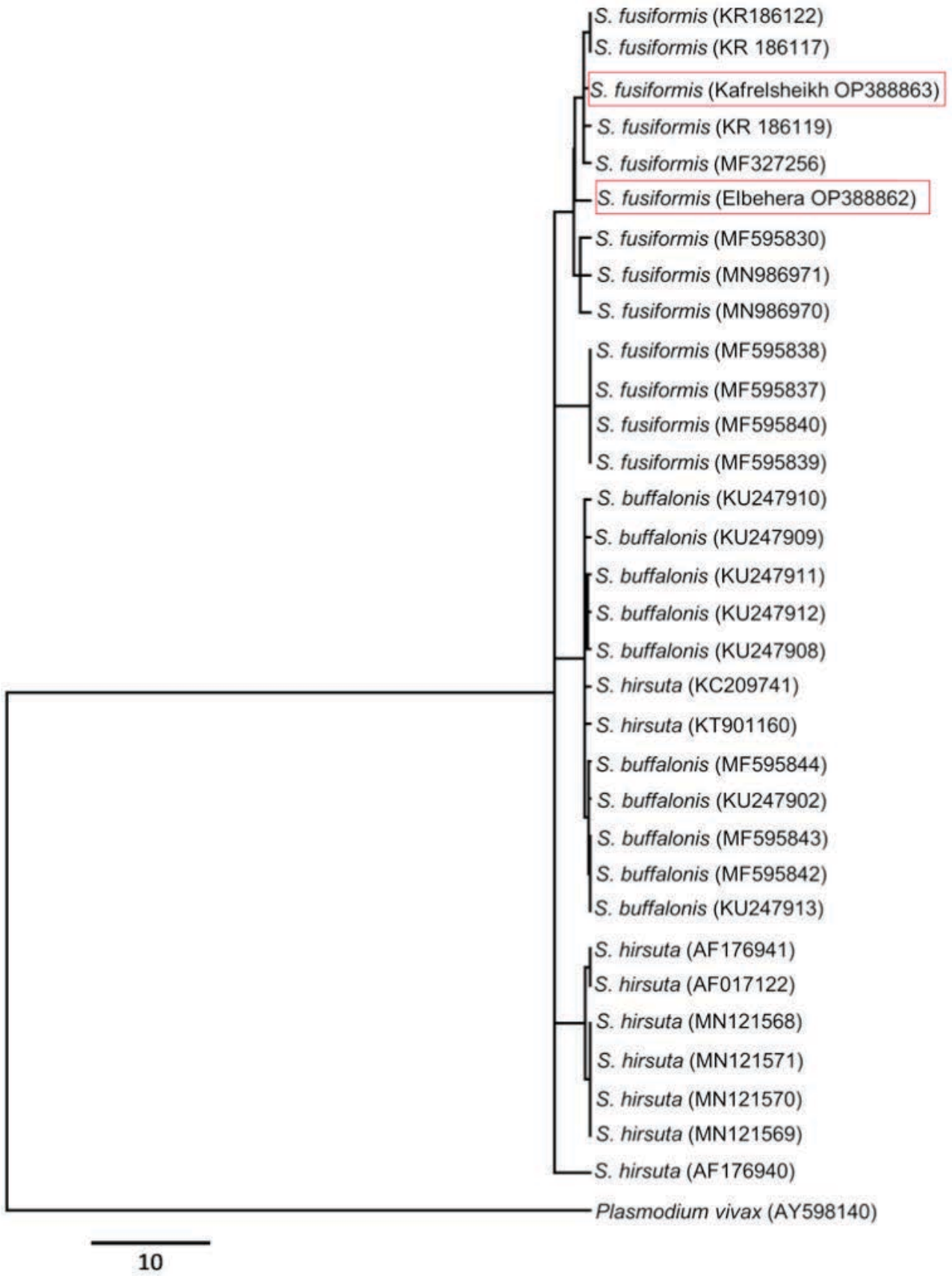

Figure 3: Phylogenetic tree of *Sarcocystis fusiformis* 18SrRNA gene from buffaloes in Elbehera and Kafrelsheikh provinces, Egypt. The tree was drawn using the Neighbor-Joining method and provided with a scale bar

69%, and 74% in Egypt (8, 28, 29). Our prevalence was higher than 41% in Vietnam (30), 22.62% in India (11), 15.6% in Iraq (31), and 8.33%, 28%, 41.5%, 42%, and 58.72% in Egypt (32, 33, 34, 17, 15). Higher prevalence of 87% in India (35) and 94%, 85.96%, and 82.3% in Egypt (36, 16, 2) were detected. Older buffaloes had a significantly higher prevalence than younger ones similar to the previous reports in Vietnam (30) and Egypt (16,17). This may be due to prolonged exposure to infections (17). Other authors reported a slight variation in prevalence in India (12) and Egypt (2). However, some owners preferred indoor rearing of young animals, so young buffaloes may be less susceptible to infection than older ones. Female buffaloes demonstrated a higher prevalence than males. This was consistent with the results reported in Egypt (32,34) and may be due to prolonged exposure to infection because of aging and exposure to stress factors like pregnancy and lactation that suppress the immune system (29). While the prevalence in males was higher than in females in Egypt (17), this may be due to the increased number of investigated males. Interestingly, gender did not affect the prevalence of *Sarcocystis* spp. infection in Iran (10, 11).

The esophageal muscles were the most infected organ, followed by the tongue. Our findings were consistent with previous studies that recorded a high prevalence of *S. fusiformis* infection in the esophagus (12, 16, 37, 17). There was no *Sarcocystis* spp. infection in the masseter muscles; in contrast with a report in Iran (10).

Histopathologically, *S. fusiformis* was embedded in the tunica muscularis of skeletal esophageal muscle and appeared elongated and large. Similar findings were reported by discovering macroscopic cysts in the esophageal muscles of water buffaloes that had thin walls filled with crescent-shaped bradyzoites (8, 28). Similarly, researchers in Malaysia (38) and Egypt (39) observed no inflammatory responses in the tissue around the cysts. The absence of an inflammatory reaction may be owing to the cysts' placement within muscle fibers, which are protected by a membrane from host immunity (40). The present study revealed normal striated muscle cells without *Sarcocystis* infection as illustrated by clear striated muscle cells and multiple peripheries located nuclei compared with infected muscle, which demonstrated detached fibers around the cyst, low number of nuclei, and disappearance of striation. This agreed with the fiber type composition of the striated muscle layer of the esophagus of the cow, sheep, donkey, dog, and cat examined with standard histochemical methods and immunohistochemical staining using type-specific anti-myosin sera (41).

18S rRNA sequencing indicated a close relationship between the locally tested isolates of Elbehera and Kafrelsheikh, with 97% identity. Elbehera and Kafr El Sheikh isolates showed sequence identity of 99.5% and 95%, and 94%, respectively, with *S. fusiformis* sequences in the GenBank, indicating that the discovered species is *S. fusiformis*. Phylogenetic analyses indicated that the two isolates were closely related to each other and the other previously registered *Sarcocystis* species in the GenBank database and were classified in the same clade. This might be related to variations in the nucleotide sequences of the *Sarcocystis* species. Furthermore, because they share the same intermediate and final host (cat), most *Sarcocystis* species are phenotypically identical (1). This finding is similar to the earlier one from Egypt (8). In conclusion, this study presented the prevalence, morphology, histopathology, and molecular characterization of *S. fusiformis* in slaughtered buffaloes in the Egyptian provinces of Elbehera and Kafrelsheikh. High *S. fusiformis* prevalence highlights the significant role of cats in *S. fusiformis* transmission and the need to adopt sanitary measures to protect animals and humans from infection such as preventing cat feces to contaminate animal feed and water and hygienic disposal of infected carcasses; furthermore, proper cleaning of the cooking utensils used to handle infected buffalo meat to protect humans.

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Authors contribution: SM, MA, MH, SSS, SIS, SFM, and BHE: conception and design, SM, MH, SSS, SIS, SFM, and BHE: acquisition of data, SM, MH, SSS, SIS, SFM, and BHE: analysis and interpretation of data, MA, SM, MH, SSS, SIS, SFM, and BHE: drafting the article, MA, SM, MH, SSS, SIS, SFM, and BHE: critically revised the manuscript for important intellectual content and final approval of the version to be published.

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