Original Article

Genotyping of *Sarcocystis* spp. in bovine carcasses from slaughterhouses in Sohag, Egypt

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ABSTRACT

Background: The prevalence of bovine sarcocystosis is high in most regions of the world. In Egypt, it constitutes a major human health problem due to consumption of insufficiently cooked bovine meat.
Objective: To determine the detection rate and species identity of *Sarcocystis* infection in slaughtered water buffaloes (*Bubalus bubalis*) in Egypt.
Material and Methods: Samples from esophagus and tongue were collected from 100 slaughtered buffaloes from different slaughterhouses in Sohag, Egypt and subjected to macroscopical, microscopical, and histopathological examinations. The genomic DNA was extracted from *Sarcocystis*-infected muscle samples and PCR–RFLP method was used to amplify partial parts of the gene encoding 18S rRNA. The PCR products were digested by restriction endonuclease enzyme BclI for species identification.
Results: Macroscopic sarcocysts were detected in 25% of slaughtered buffaloes, however, no microscopic sarcocysts were detected. Molecular analysis resulted in successful amplification of all positive samples and *S. fusiformis* was identified in all positive samples.
Conclusion: All isolates obtained from slaughtered buffaloes in Sohag, Egypt were *S. fusiformis* suggesting the potential role of cats in the transmission of *S. fusiformis*. Our study recommends strict hygienic measures to protect animals and humans from infection.

Keywords: 18S rRNA; bovine carcasses; Egypt; PCR-RFLP; *Sarcocystis* spp.; *S. fusiformis*; Sohag; water buffaloes.

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INTRODUCTION

Sarcocystis species are obligated intracellular protozoan parasites that infect human and a wide range of domestic and wild animals[1]. Its life cycle includes two hosts; definitive and intermediate host[2]. The definitive host is usually a predator (man and carnivorous animals) and the intermediate host its respective prey or herbivorous animals[3]. There are two types of human sarcocystosis distributed worldwide; human intestinal and human muscular types. Human intestinal sarcocystosis takes places due to ingestion of raw or undercooked meat with zoonotic *S. hominis*. Man becomes the definitive host with symptoms of gastroenteritis, watery diarrhea, abdominal pain, nausea, vomiting; however, the majority of infections are asymptomatic and self-limiting[4]. Water buffaloes are the intermediate hosts of *Sarcocystis* harboring macro or micro sarcocysts in their muscles. Intermediate hosts become infected via ingesting sporocysts or sometimes sporulated oocysts contaminating food or water[5].

Four characterized species of *Sarcocystis* have been reported and described in the water buffalo as the intermediate host. Two species, *i.e.*, *S. fusiformis* and *S. buffalonis* (*S. hirsuta*-like species) form macroscopic sarcocysts, with the cat as the definitive host. Two other species, *S. levinei* (*S. cruzi*-like species) and *S. dubeyi* (*S. sinensis* or *S. hominis*-like species) form microscopic sarcocysts. Dogs are the known definitive hosts for *S. levinei*, while the definitive host(s) for *S. dubeyi* has not yet been identified but is supposed to be zoonotic[6].

Esophagus, heart, tongue, diaphragm and intercostal muscles are the most common preferred organs in this infection in buffaloes and in which it persists throughout life of the host, but may start to disappear after 3 months of infection[7]. Therefore, it has great impact on public health. Beings a foodborne disease found in raw or undercooked meat, it is identified as a zoonotic parasite that causes different symptoms in humans. Correspondingly, its detection in livestock production has a very important role at the economic level, since it generates losses in the carcasses of water buffaloes and a decrease in milk production[8].

Adult *Sarcocystis* of different species is distinguishable *via* distinct phenotypic characteristics, *e.g.*, the shape, size, cyst wall thickness, *etc*. Therefore, molecular methods are considered reliable for affirming species identification and differentiation. Variable regions of the highly conserved 18S ribosomal subunit are suitable for distinguishing *Sarcocystis*

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species in hosts[9]. The PCR-RFLP based on variable regions of the small subunit ribosomal RNA sequences is considered and used widely as a rapid, inexpensive and accurate molecular approach to discriminate different protozoa as well as *Sarcocystis* spp.[5,10].

The purpose of this study was to microscopically, and molecularly investigate the detection rate and species identity of *Sarcocystis* infection in water buffaloes (*B. bubalis*) in Sohag, Egypt.

MATERIAL AND METHODS

This descriptive analytical study was conducted at Medical Parasitology Department, Faculty of Medicine, Sohag University during the period from January 2023 to April 2024.

Study area: The study was conducted in Sohag Governorate, Upper Egypt. Sohag is situated in the southern region of the country about 467 kilometers to the south of Cairo. It extends into the Nile Valley with a total size of 1547 km^2.

Study design: Samples of esophagus and tongue were collected from 100 freshly slaughtered buffaloes of different ages and sexes from different slaughter houses in Sohag, Egypt. All samples were transported in an icebox to the laboratory for macroscopic, microscopic, and histological examinations as well as molecular analysis.

Macroscopic examination: The macroscopic sarcocysts detected by gross naked eye inspection, palpation and incision of the muscular tissues appeared whitish with different shapes[4].

Microscopic examination: Small slices of the muscle tissues were compressed between two glass slides (impression technique), stained with Giemsa and examined by light microscope (400×) for presence of microscopic sarcocysts[11].

Histological examination: Specimens from positive muscular samples were fixed in 10% formalin and processed for histopathology. The samples were subsequently stained with hematoxylin and eosin and microscopically examined[12].

Extraction of DNA: The samples which were microscopically positive for sarcocyst were preserved at -20°C for DNA analysis. The genomic DNA was extracted using Genomic DNA Mini extraction kit (Applied Biotechnology) according to the manufacturer instructions. The extracted DNA was stored at -20°C for PCR amplification[9].

Samples amplification: Partial sequence of the *18S rRNA* gene of *Sarcocystis* spp. was amplified by PCR using the following pair of primers: forward primer sar F 5/CGT GGT AAT TCT ATG GCT AAT ACA 3/ and reverse primer sar R 5/ TTT ATG GTT AAG ACT ACG ACG GTA 3 as described by Yang *et al*.[13] and Hooshyar *et al*.[14]. The amplified fragment size was approximately 900 bp. The PCR reactions were carried out in a total volume of 25 µl, including 12.5 µl of COSMO PCR RED Master Mix kit (Willowfort, UK), 0.5 µl of each primer of 20 pmol concentrations, 6.5 µl of nuclease free water and 5 µl of DNA. The PCR reactions were conducted on Thermal Cycler (Veriti, Applied Biosystems, USA) with the following cycling conditions: an initial denaturation step at 94°C for 5 min and 35 cycles at 94°C for 30 sec (denaturation), 58°C for 1 min (annealing), 72°C for 1 min (extension) with a final extension step for 5 min at 72°C. For each PCR set, negative control (no-DNA) was included. Products were separated by electrophoresis on 1.5% agarose gel, stained by ethidium bromide and then visualized under ultraviolet (UV) light. A 100 bp DNA ladder was used as a size marker[15].

Protocol of RFLP: The PCR products were digested with the restriction endonuclease enzyme BclI (Jena Bioscience, Germany) to distinguish between species. The RFLP digestion with this enzyme results in fragments of 358 and 595 bp lengths for *S. hirsute*-like and 782 and 140 bp lengths for *S. hominis*-like. *S. fusiformis* remains uncut with BclI enzyme. For RFLP analysis: 8 µl of PCR product, 5 µl distilled water, 1 µl (10 U/lL) restriction enzyme and 1 µl buffer were added into a total of 15 µl reaction mixture and incubated for 3 h at 55°C. The fragments (10 µl) were detected in 1.5% agarose gel electrophoresis (90 volts for 60 min) and visualized by UV Transilluminator Equipment. A 100 bp DNA ladder was used to estimate the sizes of the restriction fragments[15,16].

Ethical considerations: This study was approved by the Institutional Animal Care and Use Committee (IACUC) of Sohag University under approval certificate number Sohag-IACUC/5/16/2022/01 and approved on November 2022.

RESULTS

The current study revealed that the overall infection rate of *Sarcocystis* spp. in slaughtered water buffaloes was 25%. Further, macroscopic sarcocysts were detected but no microscopic sarcocysts were identified. The macroscopic sarcocysts consisted of opaque, spindle or fusiform shaped bodies, milky white in color and ranging in size from 5-15 mm long x 1-4 mm wide (Fig. 1). Microscopic examination of histological section showed a smooth thin cyst wall surrounding a cyst cavity divided into typical compartments of different sizes by thin septa. Two parasitic stages were within the cysts, metrocytes that acquired a pale stain near the margin of the cyst and bradyzoites condensed at the periphery then decreased towards the lumen (Fig. 2).

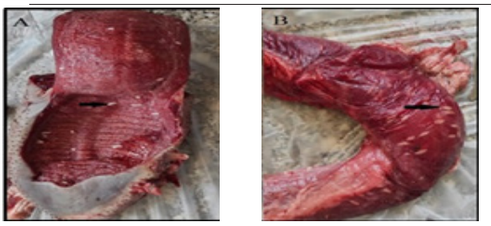

Fig 1. (A): Tongue of slaughtered buffaloes infected with many macroscopic sarcocysts between the muscle bundles (arrow). **(B):** Esophagus of slaughtered buffaloes infected with numerous macroscopic sarcocysts distributed mainly under the serosal membrane (arrow).

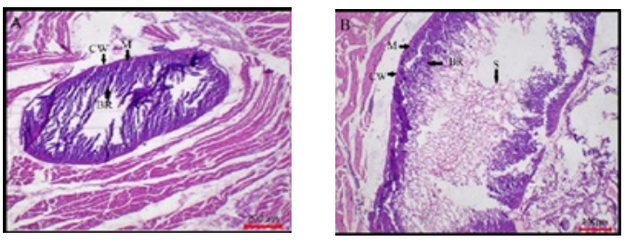

Fig 2. Histopathological sections stained with H&E representing **(A):** macroscopic sarcocyst within tongue muscle of infected buffalo. **(B):** Macroscopic sarcocyst within esophageal muscle of infected buffalo. Thin sarcocyst wall **(CW)**, septa **(S)**, groups of bradyzoites **(Br)**, and faint staining metrocytes **(M)**. (Scale bar=200 µm).

The PCR analysis, revealed that macrocysts samples produced DNA fragments of approximately 900 bp on agarose electrophoresis gels indicating the existence of *Sarcocystis* spp. (Figure 3). The RFLP analysis of PCR products showed that the restriction endonuclease enzyme BclI did not result in any digestion band pattern of the *18S rRNA* gene and hence all positive *Sarcocystis* samples represented *S. fusiformis* (Figure 4).

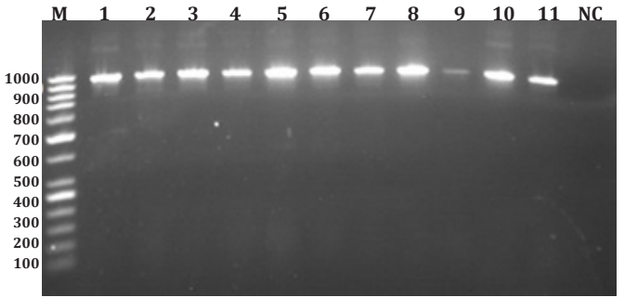

Fig. 3. Agarose gel electrophoresis image showing PCR products of *18S rRNA* gene of *Sarcocystis* spp. **Lane M:** 100 bp DNA ladder; **lanes 1–11:** positive samples (approximately 900 bp); **lane NC:** negative control.

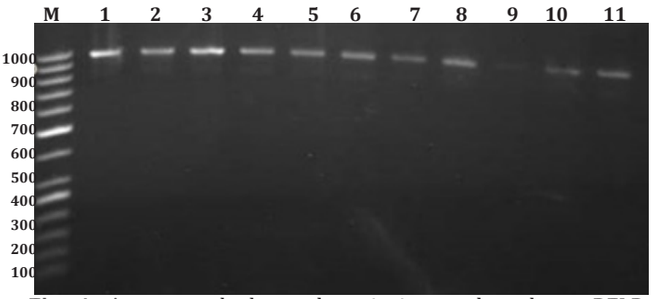

Fig. 4. Agarose gel electrophoresis image that shows RFLP pattern of PCR products with BclI. **Lane M:** 100-bp DNA ladder, lanes **1-11:** *S. fusiformis* (undigested PCR product).

DISCUSSION

Traditional methods for identification and characterization of *Sarcocystis* spp. are based on isolated cyst morphology using gross examination and light microscopy. However, molecular methods were found useful and sensitive in identifying the different *Sarcocystis* spp. Additionally, molecular techniques can be utilized to distinguish the morphologically comparable *Sarcocystis* cyst in intermediate hosts if it is the same or belongs to different species[17]. In the present study, the overall detection rate of macroscopic sarcocysts among slaughtered buffaloes was 25%. The results nearly agreed with those obtained by Khalifa *et al*.[18] who reported an infection rate of 28 % macroscopic sarcocysts in buffaloes in Sohag, and those by Metwally *et al*.[19] who found the infection rate of 25% using macroscopical examination and 27.7% using microscopical examination in buffaloes in Assiut. Moreover, results were close to those by Aziz *et al*.[20] who reported that 26.9% of buffaloes muscle samples were positive by routine abattoir examination while 52.4% were positive by microscopic examination in Sohag. From Tanta abattoir, Gerab *et al*.[6] detected 26.5% animals positive for macroscopic sarcocysts and 56% positive for microscopic sarcocysts. This suggested that buffaloes are exposed to infection due to close contact with dogs, cats and even wild animals that act as final hosts for these protozoa.

Our results were lower than those recorded in Egypt by El-Dakhly *et al*.[21] who reported 78.9% of macroscopic and microscopic sarcocysts in Beni-suef. Abu-Elwafa *et al*.[22] also detected 58.7% of macroscopic sarcocysts in Dakahlia. In Cairo, Ibrahim *et al*.[4] reported that while 48.6%-63.2% of macroscopic and microscopic sarcocysts were detected in old buffaloes, 41.2-53.8% were detected in young ones. Higher results (41.50%, 75.3%, and 65.9%) of macroscopic sarcocysts

were recorded also in Sharkia[23], Elbehera[24] and Kafrelsheikh[24], respectively. Similar higher results were recorded in other countries such as Punjab, India where Dar *et al*.[25] reported 95.5% of microscopic cysts, and 0.60 % of mixed infection with macroscopic cysts. Likewise, JyothiSree *et al*.[7] found a total of 66.42% with 22.62% macroscopic cysts and 43.79% microscopic cysts in Andhra Pradesh India. Dameshghi *et al*.[9] also revealed 73.25% of microscopic cysts and 1.25% of macroscopic cysts in Guilan, Iran. On the other hand, our results were higher than those reported in El-Kharga (8.33%)[13]. The difference in detection rates may be due to the different methods of diagnosis, different localities, different climates and different management practices that affect the survival of cysts in these environments.

In the present study, the PCR-RFLP analysis showed that *S. fusiformis* was identified in all positive *Sarcocystis* samples using the restriction endonuclease enzyme BclI. This uniquity of infection indicates that it is due to exposure to the same definitive host which in this case is the infected cat. The buffaloes drink water and eat feed contaminated with sporocysts voided by the infected cats. Our results agreed with those deduced by Khalifa *et al*.[18] who reported that only the macroscopic fusiform-shaped species was detected in Sohag using microscopical, histopathological and transmission electron microscope examinations. The findings were also in accordance with other previous studies in Egypt. Ras[23] reported that the isolates obtained from buffaloes were macroscopic *S. fusiformis* in Sharkia using both morphological and nucleotide sequence analysis of the gene encoding *18S rRNA*. Menshawy *et al*.[24] by its sequencing analysis revealed that the recovered species from buffaloes were *S. fusiformis* in Elbehera and Kafrelsheikh.

Furthermore, our findings agreed with previous studies in Khuzestan, Iran by Oryan *et al*.[16] where PCR-RFLP analysis using four restriction enzymes (Dra1, Ssp1, Fok1 and BclI), revealed that all positive *Sarcocystis* samples in buffaloes represented *S. fusiformis*. Hamidinejat *et al*.[5] identified *S. fusiformis* in water buffaloes using restricted enzymes (SspI and DraI). We attributed this difference to the fact that the isolated samples were mostly microscopic cysts, while our study samples were macroscopic cysts. Although *S. fusiformis* is considered nonpathogenic but its production of macrocysts effects meat quality predisposing to economic losses[5].

In conclusion, the present morphological and genetic study of *S. fusiformis* in slaughtered buffaloes in Sohag provided useful information for monitoring and controlling of *Sarcocystis* infections. The high *S. fusiformis* prevalence indicates the significant role of cats in *S. fusiformis* transmission and the need to adopt sanitary measures to protect animals and humans from infection. This entails preventing cat feces to contaminate animal feed and water and hygienic disposal of infected carcasses; furthermore, it is very essential to educate the public about the zoonotic aspect of this disease and its transmission.

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