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Microscopic and IFAT Based Incidence of Sarcocystis in Naturally Infected Buffaloes (*Bubalus bubalis*) at Sohag, Egypt

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Abstract
The aim of the present work was to detect the incidence rate of *Sarcocystis* spp. from Egyptian Water Buffaloes (*Bubalus bubalis*) in Sohag governorate, Egypt. Blood and muscle samples (145 each) were collected from neighborhood abattoirs. *S. buffalonis* 33.8%, *S. levinei* 14.48% and *S. fusiformis* 0.41% were recognized by microscopic examination and confirmed by indirect immunofluorescence antibody technique (IFAT). Results showed that 26.9% examined samples were positive by routine abattoir examination while 52.4% examined samples were positive by microscopic examination, on the other hand; 64.8% samples were positive by IFAT. We can concluded that, gross examination at the abattoirs is not adequate to present safe meat for human utilization, and it must be supported with other serological tests like IFAT, although it has a moderate specificity and high sensitivity to diagnose Sarcocystosis.
Keywords: Buffaloes, Sarcocystis, IFAT, Sohag, *S. buffalonis*, *S. levinei*, *S. fusiformis*, Egypt.

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INTRODUCTION

Sarcocystis is a protozoan genus of parasites, the majority of species infecting muscles of several species of domestic animals, as an intermediate host to form the cyst, but dogs, cats, and human act as a final host. *Sarcocystis* spp. are exceptionally common in animals and are thought to be host specific, the most vital zoonotic parasites and have public health significance through meat utilization (Dubey *et al*., 1989).

Some of *Sarcocystis* species have a pathogenic effect on their host causing severe economic loss such as decrease of weight, low feed conversion ratio, fever, weakness of muscles, decreased milk output, some cases of late abortion and may be death of carrier animals (El-Morsey, 2010; El-Morsey *et al*., 2014). The gross visible cysts, as *S. fusiformis*, *S. buffalonis* and *S. gigantea*, make the meat unaesthetic and thus lead to partial or total condemnation of infected carcasses. Until now, four characterized species of *Sarcocystis* have been distinguished in the water buffaloes as *S. fusiformis, S. buffalonis, S. levinei* and *S. dubeyi* (Hilali *et al*., 2011). There are various reports about the prevalence of other parasites in naturally infected water buffaloes (Iqbal *et al.,* 2013; Iqbal *et al.,* 2014a; Iqbal *et al.,* 2014b; Muhammad *et al.,* 2015; Ojeda-Robertos *et al.,* 2017).

Water buffaloes are usually slaughtered in local abattoirs in Sohag province, therefore, the perfect meat inspection and identification of such zoonotic parasite is essential to prevent its danger. Veterinary inspection in abattoirs is extremely important either economic or public health point of view because a great amount of visceras is rejected in order to maintain a low risk for human (Vilallonga and Valcarcel, 2016). There is only one published scientific work on the incidence of *Sarcocystis* spp. in Egyptian water buffaloes in Sohag province (Khalifa *et al*., 2008). Therefore, the aim of the present work was to determine the incidence of *Sarcocystis* spp. in Egyptian water buffaloes in Sohag province.

MATERIALS AND METHODS

Ethical Considerations

The Study protocol was reviewed and approved by the ethics committee of the Faculty of Veterinary Medicine, Sohag University on October 14, 2014**.** The animals from which *Sarcocystis* spp. were collected were being processed at a local abattoir in Sohag Province, Egypt, as part of the normal work of the abattoir.

Study and sample collection

Sohag province located in the southern part of Egypt (Figure 1) with Coordinates of 26°33′N, 31°42′E with Longitude of (31.694780), and Latitude (26.556950), it is elevated 67m over sea level (Wikipedia, 2017). A total of 145 blood and 145 muscle samples from esophagus, diaphragm, masseter, and shoulder from freshly slaughtered buffaloes in local abattoirs were collected from January to May 2017. Samples were labeled and submitted to the department of Parasitology, Faculty of Veterinary Medicine, Sohag University, Egypt, preserved in labeled ice bags and kept refrigerated till further work.

Fig.1. Sohag province located in Upper Egypt

Microscopic examination

The collected 145 muscle samples from different parts of the carcass were grossly examined by the naked eye for the presence of any cyst. Positive tissue specimens were fixed in neutral buffer formalin, dehydrated in ascending grades of ethyl alcohol, cleared and embedded in paraffin blocks. Sections of 5-7µm were made and stained with H and E stain (Bancroft and Stevens, 1993) and examined at (40 x) by light microscope, measurements in µm were detected by fotosizer program.

Indirect immunofluorescence antibody technique (IFAT)

A total of 145 blood samples collected from slaughtered buffaloes were centrifuged at 2500 rpm for 10 minutes and serum samples were stored at -20°C until use for IFAT. Positive and negative control sera of *S. fusiformis* of buffaloes were obtained friendly from Rasha A. El Meghanawy, Animal Health Research Institute, Menofia, Egypt. Macroscopic cysts were collected, cracked and every 1 ml of the liquid was diluted by 3 ml of physiological saline (0.9 %) and utilized as antigen. 25 µl of the antigen was added on immunofluorescent slides and left to air drying, fixed by cold acetone washed by Phosphate Buffer Saline (PBS) and left to air drying. Serum was diluted by sterile PBS to (1:16), 25 µl of the diluted serum was added to the fixed antigen on the tissue slide, then put in humid condition in the incubator for 30 minutes at 37°C, and washed 3 progressive times by PBS three minutes each. 10 µl from the anti-human conjugate was added to 2ml of 1% BSA (Bovine Serum Albumin), and 10 µl from the anti-bovine conjugate was added to 2ml of 1% BSA, then 25 µl of each diluted conjugate was added to the slides. The slides left in the incubator for 30 minutes at 37°C and then mounting buffer was added, slides were examined with the fluorescent microscope at magnification 400X and photographed **(**El-Nazer and Abdel-Azem,2000; Portella *et al.,* 2016).

Statistical analysis

Data obtained was tabulated using Microsoft Excel (MS Excel 2010, Microsoft Corporation). SPSS version 16.0 statistical software (SPSS, Chicago, IL) was used for statistical analysis following Chi-square test. Sensitivity and specificity was calculated following Savini et al. (1994) as in table 1.

Table 1. Method of calculation the different evaluation parameters of the tests

Macroscopic	IFAT		Total
	Present	Absent	
Positive	(a)	(b)	(a+b)
Negative	(c)	(d)	(c+d)
Total	(a+c)	(b+d)	(n)

a: True positive samples which positive by macroscopic and IFAT, b: False positive is negative by macroscopic but positive by IFAT, c: False negative which were positive macroscopically but negative by IFAT, d: True negative: samples negative by macroscopically and IFAT.

The sensitivity of the test = a/(a+c) x 100;
Specificity of test = d/(b+d) x 100;
Positive predictive value = a / (a+b) x100;
Negative predictive value = d / (c+d) x 100;
Predictive validity value = (a+d)/n x 100.

RESULTS

The results of gross examination by eyes of 145 samples showed 39 positive cases (26.9%) with *Sarcocystis*, while histopathological examination showed 76 positive cases (52.4%), and 94/145 (64.8%) were positive by (IFAT) (Table 2). Three species: *S. buffalonis* 33.8%, *S. fusiformis* 14.48% *S. levinei* 0.41% were distinguished (Table 3). *S. fusiformis* were found located

subserosal parallel to longitudinal axis between muscle filaments (Figure 2 and 3) while *S. buffalonis* were long and curved in shape, milky white colored cysts, and located immersed in the connective tissue along the longitudinal axis of muscle fiber (Figures 4 and 5). *S. levinei* were not seen by naked eyes, they were spherical in cross section or ovoid in longitudinal section (Figure 6: a, b). Histopathological examinations revealed the presence of myositis in form of degenerated and necrosed muscle fibers and diffuse mononuclear cell infiltration of lymphocytes and eosinophils in the connective tissue of the skeletal muscles where *Sarcocystis* were present (Figure 7). Results of IFAT revealed that 94 out of 145 (64.8 %) examined serum samples had antibodies against *S. fusiformis*, (Table 2). All 39 samples with macroscopic cyst of *S. fusiformis* were IFAT positive (100%), which showed diffuse fluorescent pigment in the trophozoites in the dark background (Figure 8). Sensitivity was (100%), specificity (48.11%), Positive predictive value (41.49 %), Negative predictive value (100%) and Predictive validity value was 62% (Table 4).

Table 2. Incidence of Sarcocystis in the examined buffaloes in Sohag.

Examined buffaloes N=145	Macroscopical		Histopathological		IFAT	
	No.	%	No.	%	No.	%
Positive	**39**	**26.9**	**76**	**52.4**	**94**	**64.8**

Table 3. Incidence of the identified *Sarcocystis* spp. in the examined buffaloes by histopathological examination (over all 76 positive out of 145 examined samples).

Organ	*S. buffalonis*		*S. fusiformis*		*S. levinei*	
	Positive	%	Positive	%	Positive	%
Esophagus	36/145	24.82	12/145	8.27	4/145	0.27
Tongue	13/145	8.96	9/145	6.20	2/145	0.14
Total	49/145	33.8	21/145	14.48	6/145	0.41

Table 4. Evaluation of IFAT in diagnosis of *Sarcocystis* spp. in buffaloes.

IFAT				Evaluation parameters %				
T.P	T.N	F.P	F.N	Sensitivity	Specificity	P.P.V	N.P.V	P.V.V
39	**51**	**55**	**0**	**100**	**48.11**	**41.49**	**100**	**62**

T.P: true positive; T.N: true negative; F.P: false positive; F.N: false negative; P.P.V: Positive predictive value; N.P.V: Negative predictive value; P.V.V: Predictive validity value.

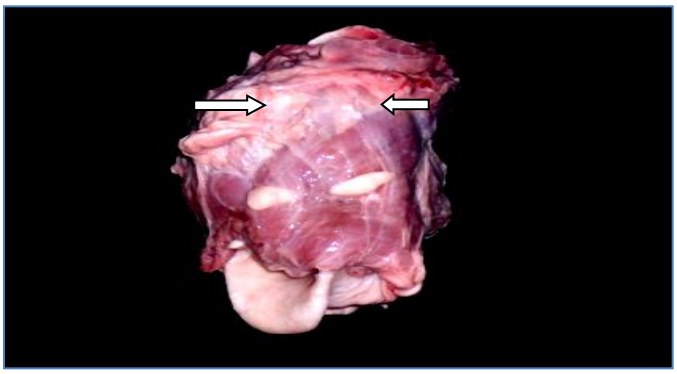

Fig. 2. Photomicrograph showing esophageal muscle infested with *Sarcocystis fusiformis*, Spindle or elliptical in shape and lying subserosal parallel to the longitudinal axis of the esophageal muscle fibers (white arrows).

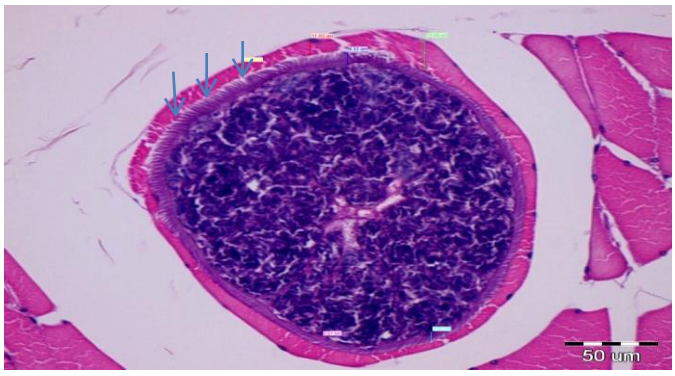

Fig. 3. Photomicrograph showing *S. fusiformis*, it is spherical or sub spherical and measured from (219.9 x 223.11 µm), wall thickness (1.82 - 2.73 µm), formed from a long striated projections in a palisade-like manners (blue arrows) measured from (8.32-8.90µm) and the cyst was partitioned with septa into several sporadic irregular

compartments loaded with bradyzoites. The bradyzoites were highly condensed measured from (9.44-13.46 µm), seen in the histopathologicalcross section in esophageal muscle fibers. H&E (bar size is 50 µm).

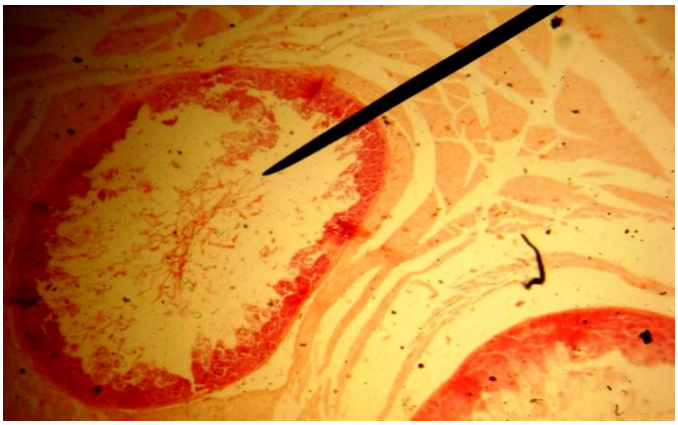

Fig. 4. Photomicrograph showing cross sections in *S. buffaloni*, thick wall overcrowding by bradyzoites behind the wall, while the center of the cyst is free from bradyzoites (arrow) H&E (X40).

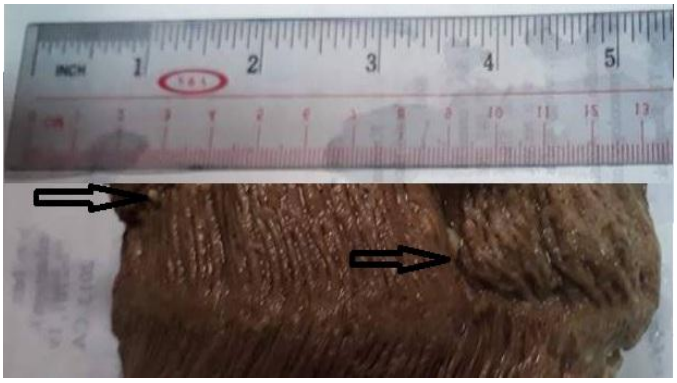

Fig. 5. Photomicrograph showing longitudinal sections of tongue muscle infested by *S. buffaloni,* creamy white or milky in color (black arrow)

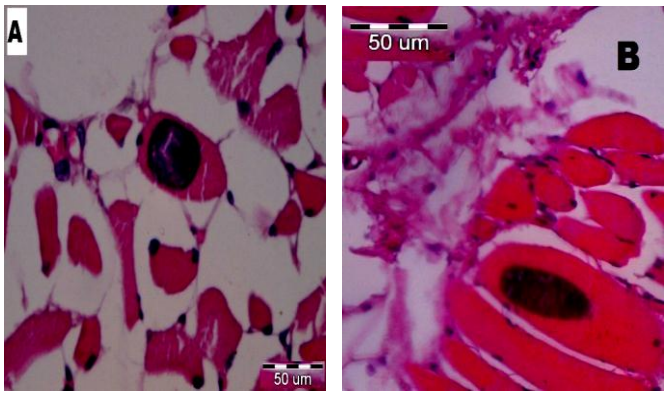

Fig. 6. Photomicrograph showing (A) cross section and (B) longitudinal section of microscopic cyst *S. levinei*, very thin wall and fine septa with several compartments, center, and periphery are highly condensed with bradyzoites. Seen in tongue muscle fibers H&E (bar size is 50 µm).

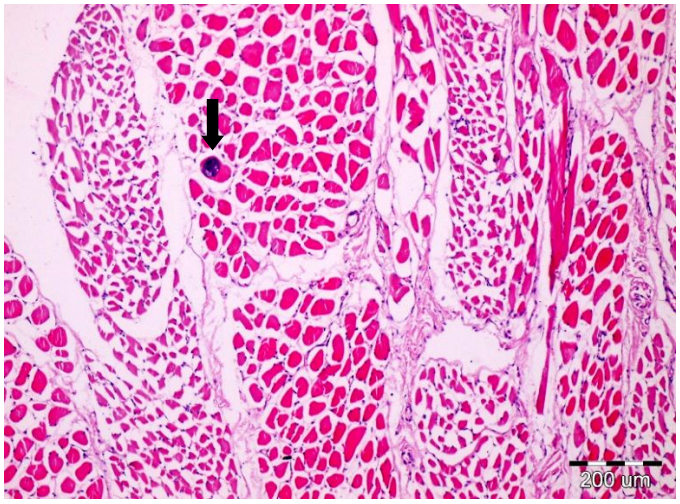

Fig. 7. Photomicrograph showing eosinophilic myositis (glossitis), capsulated Sarcocystis (black arrow) surrounded by degenerative and necrotic muscle fibers, and diffuse mononuclear cell infiltration of lymphocytes and eosinophils seen in the connective tissue of tongue skeletal muscles. H&E stains (bar size 200µm).

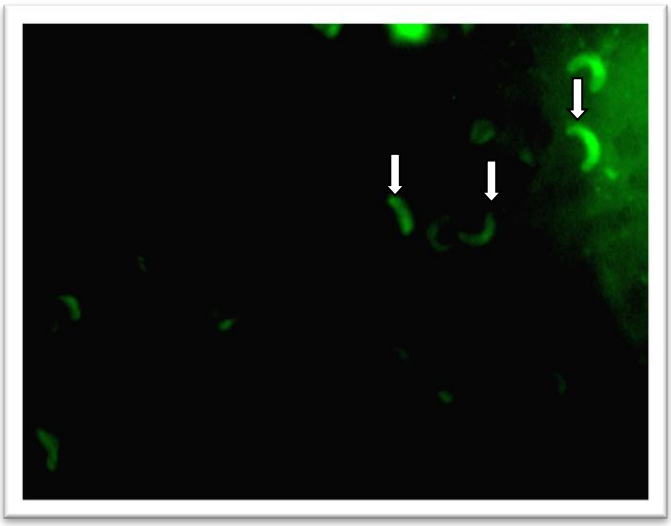

Fig. 8. Photomicrograph showing Positive IFAT in serum samples as a green diffuse fluorescent pigment in the tachyzoites (white arrows)

DISCUSSION

Several studies were carried out in Egypt on the prevalence of *Sarcocystis* spp. of water buffaloes, in Sohag by Khalifa et al. (2008); Assiut by Metwally et al. (2014), Abdel Rahman (2001); Said (1996), and Fatma et al. (2008); Qena by Fawaz (1998); Beni-Suef, by El-Dakhly et al. (2011); Dakahlia Province by Abu-Elwafa et al. (2015**);** in Cairo by Hilali et al. (2011; Behera province by Hendawy (2006) and in Kafr Elshiekh by El-Seify et al. (2014). Water buffalo are susceptible to most diseases and parasites affecting cattle. The current work was done to detect the incidence rate of infection of *Sarcocystis* spp in Egyptian water buffaloes at Sohag province. There was only one study by Khalifa et al., (2008), who revealed three species *S. cruzi, S. hominis, and S. fusiformis* in water buffaloes. In the present work, histopathological and IFAT test as serological test, sensitivity and specificity were detected, results of the present study come nearly in agreement with those of Ashmawy et al. (2014) who revealed the prevalence of *Sarcocystis* spp. infection to be 67.6 % in buffaloes aged 5-7 years old, and Wahba (1994) who recorded 73% in buffaloes slaughtered at Cairo and Belbis slaughter houses, Egypt. This suggests that buffaloes may be exposed to infection due to their close relationship with dogs, cats and even wild animals that act as final hosts for these protozoa. A higher incidence (93 %) was recorded among water buffaloes aged over 5 years old in Behera Province, Egypt (Hendawy 2006). The difference in incidence may be due to the different methods of diagnosis, different localities, and different management prcatices. The common serological test for *Sarcocystis* spp. detection by researcher is the enzyme-linked immunosorbent assay (ELISA) test due to its high sensitivity and specificity values (Savini et al. 1994; Zayed and El-Metenawy, 2002; Hendawy 2006; Ashmawy *et al*., 2014; Metwally *et al.,* 2014). The present study proved that IFAT test can also be considered a reliable diagnostic test for *Sarcocystis* spp. infection in water buffaloes. There is only one previous study by El Nazer and Abdel-Azem (2000), they used *S. fusiformis* antigen in ELISA and IFAT for detection of extra intestinal sarcocystosis in human in attendants of rheumatology clinic in Sohag University Hospital, this may be due to the concept of Tadros et al. (1981) who found a remarkable degree of cross reaction among *Sarcocystis* species from widely divergent host origins.

There is a high variation of infection rate at different localities in Egypt among examined animals, this high rate of infection may be due to the lack of therapeutic courses against sarcocystosis is of little or no value either for the tachyzoites or muscle cyst; involvement of several final hosts in completion of the cycle; main output of sporocysts as infective form for long periods; oocysts or sporocysts resistance to severe environmental conditions as desiccation, drying, in addition to little or no immunity to shedding of sporocysts.

CONCLUSION

In conclusion, there are three *Sarcocystis* spp. infecting water buffaloes in Sohag province, Egypt and IFAT test has mild ability to predict the presence of macroscopic cyst. In addition, this study proves that the routine examination at abattoirs is not sufficient to introduce safe food for human consumption, and must be combined with other serological tests as IFAT. Our findings prove the hypothesis that the antigen of *S. fusiformis* has cross antigenicity to other *Sarcocystis* spp. with mild specificity and very high sensitivity to detect sarcocystosis.

AUTHORS' CONTRIBUTIONS

Amer Ragheb Abdel Aziz conceived the research, wrote the first draft, contributed to data analysis and interpretation, and revised the manuscript, also sample collection, preservation, and labeling. Fatma Abo Zakaib Ali, and Doaa Salman worked on sample histopathological sections and performed diagnostic measurements. Amer Ragheb Abdel Aziz made the final identification of *Sarcocystis* spp. Amer Ragheb Adel Aziz, and Doaa Salman performed IFAT test and interpretation. All authors made the data analysis and interpretation, revised the manuscript approved the final version of the manuscript.

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CONFLICT OF INTEREST

The writers have announced that no contending interest exists.

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