Prevalence of Sarcocyst in Egyptian Buffaloes Slaughtered at El-Menofia Abattoirs

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A B S T R A C T

Sarcoctosis is a zoonotic disease invading the muscles of domestic animals caused by sarcocystis species, acyst forming coccidian parasite in phylum apicomplexa. Sarcocystis species are obligatory intracellular protozoa with a typical coccidian life cycle consisting of merogny, gametogny and sporogny. These parasites have indirect life cycle, cycling between two host involving carnivores as definitive host, where intestinal infection occur and herbivorous or omnivorous as intermediate host, where tissue invasion occur. A total of 937 buffaloes (130 males under 2 years and 807 females above 5 years) were examined during a year from November 2014 till October 2015. in Shebin El-kom abattoir, El-Shohada abattoir and El-Batanon abattoir, El-Menoufia Governate, Egypt for detection of macroscopic and microscopic sarcocystis cysts in muscular tissues (esophagus, masseter muscles, tongue and pharyngeal muscle) of slaughtered animals. The results indicated that the muscle samples revealed the infestation rate with macroscopic S. fusiformis in buffaloes were 82.92% as general percent, but in animals over 5 years it was 92.81% and in animals under 2 years it was 21.54% the esophagus, tongue and the diaphragm were found to be the most common sites for Sarcocystis species. Results also indicated mixed infections with both macroscopic S. fusiformis and both S. bovini and S. silva. This was the first record for them in Egypt. Also identification of sarcocystis species by using PCR revealed 3 species S. fusiformis, S. bovini and S. silva.

Keyword: Sarcosyst, Bufaloes, Abattoir and PCR.

1. INTRODUCTION

The water buffaloes (Bubalus bublis) are the most important domestic animals in Egypt as they are considered the most preferable animals to the farmers as they produce milk of high fat content. Although buffaloes generally are of good health condition, they are susceptible to the most parasitic diseases that affect cattle. Sarcocytosis is one of the most prevalent parasitic infections of buffaloes. Meat intended for human consumption must be free from this parasite due to its zoonotic importance (in some species), rather than unpalatability of such meat because of the cysts specially if calcified, resemble the grain of sand (Liu et. al, 2009). No doubt that the all carcasses value is in the muscles so, the muscle diseases are of primary importance to the meat producing and meat packing industries. The most important problem of imported buffalo meat to Egypt from India is sarcocytosis due to its unmarketability. Sarcocytosis also causes losses, due to condemnation of organs after slaughter (Claro and Romeo, 2008). Most Sarcocystis species infect specific hosts or closely related host species (Fayer, 2004). Unlike other species of Coccidia, sarcocystis is shed in the faeces in the infective form. That is, the oocyst does not depend on weather conditions to sporulate and hence is infective. The cysts vary in size from a few micrometers to several centimeters, depending on the host and species (Merck, 2005). Humans can serve both as intermediate and definitive host (Fayer, 2004). Sarcocystis shown to be a zoonotic parasite when humans consume infected meat and accidental ingestion of oocyst/sporocysts from infected animals. Locally known predators such as dogs, cats and snakes could excrete infectious sporocysts that may find their way through contaminated water or food, eventually infecting man. Therefore, they remain potential but unknown sources of human intestinal sarcocytosis (Fayer, 2004). In the absence of effective meat inspection, consumers of beef and pork are likely to be infected with Sarcocystis. Mature sarcocyt of each species varies in size from microscopic to macroscopic, however these features vary with the stage of Sarcocystis species and the host cell type.
(Yang et al., 2002). Human intestinal Sarcocystosis is a zoonotic disease caused by two coccidians sarcocystis hominis (S. bovihominis) due to consumption of raw infected beef and Sarcocystis meischeriana (S. suihomnis) due consumption of raw infected pork (Bunyaratvej et al., 2007). Therefore, the present study was planned out to detect sarcocysts from fresh meat collected from different abattoirs at El-Menofia Governorate (Shebin-El-Kom abattoir, El-Shohada abattoir and El-Batanoon abattoir). This work aimed to determine the prevalence and Molecular characterization of Sarcocystis fusiformis cysts in the musculature of water buffaloes in El-Menofia abattoirs (Shebin El-Kom –El-shohada and El-Batanon abattoir) in Egypt.

2. MATERIAL AND METHODS

2.1. Collection of samples:

A total of 937 buffaloe carcasses 807 female>5 years and 130 males < 2 years were subjected to post mortem inspection during a year from November 2014 till October 2015. Carcasses were collected Shebin El-kom –El-shohada and El-Batanon abattoirs.

2.2. Macroscopic examination:

Carcasses were inspected grossly by naked eye for the detection of macroscopic forms of sarcocystis species (S. fusiformis, S. buffalinos). The esophageal, pharyngeal, tongue, diaphragmatic and masseter muscles were carefully inspected for the presence of macroscopic forms of sarcocystis cyst. Samples were collected from these muscles in addition to the carcass muscles in the light and heavy infestation for histopathological and PCR examination.

2.3. Collection of Sarcocystis cysts:

Buffaloes esophageal muscles, pharyngeal muscles, diaphragmatic muscles, neck muscles, intercostal muscles and tongue muscles infected with macroscopic sarcocystis cysts were taken as soon as animal were slaughtered then washed several times with running water, then were carefully excised with the help of sharp scalpel and forceps to remove the sarcocystis cysts carefully from the surrounding tissues without injuring the cyst wall. The collected cysts were immediately washed several times using chilled 0.85 normal saline (Khulbe et al., 1989) and stored at -20°C in phosphate buffer saline (PBS) till use, these samples were transferred aseptically to the laboratory for further examination.

2.4. Collection of muscle samples apparently free from Sarcocystis cysts:

The muscle samples were taken from the fresh esophagus, masseter muscles, tongue, and pharyngeal muscles of apparently healthy animals as soon as animals were slaughtered then washed several times with running water for microscopical examination.

2.5. Microscopic examination:

After collection of muscle samples from each organ the positive parasitic lesions were examined histopathologically to determine the changes in the infested tissues. The specimens for histopathological examination, the muscle samples were shipped and examined grossly and under a stereomicroscope at 20–30 magnification. Macroscopic cysts were excised from the musculature using a fine needle. The needle was rinsed in distilled water between each cyst isolated from the same animal and new needles were used for every new animal examined. All cysts were classified according to their morphology. The dimensions of the macroscopic sarcocysts that were easy to identify under the stereomicroscope were classified in situ based on their characteristic size, shape and location. Sarcocysts that could not be identified in situ were excised and then examined for characteristic surface protrusions under a light microscope at 10X magnification. Individual isolated cysts were placed in 1.5 ml Eppendorf tubes with 20 ml distilled water. Several cysts of each species were isolated from the tissue of each animal and stored frozen at -20°C until DNA isolation (Dahlgren. and Gjerde 2007a). Species identification followed (Odening et al., 1995 and 1996) and (Dubey et al., 1989). Merozoites were released and genomic DNA was isolated by proteinase K and 0.25% trypsin digestion. Total DNA was extracted by the phenol/chloroform method.

2.6. Examination of the muscle samples for detection of the microscopic sarcocystis species:

For detection of microscopic species, histological examination of 5 standard esophageal muscle, 5 pharyngeal muscle, 5 tongue muscles, 3 neck muscle, 4 diaphragmatic muscle samples, 3 abdominal muscle (5 mm x 10 mm each) were obtained, fixed in neutral formalin 10%, sectioned at 5 microns, stained with hematoxyline and eosin (H and E) and examined by light microscopy at magnification X 40 and X 100. Also we examine inflamed inguinal lymph node of buffaloes infected with sarcocystosis for detection of the effect of sarcocysts.

2.7. PCR examination:
Prevalence of Sarcocyst in Egyptian Buffaloes Slaughtered at El-Menofia Abattoirs

Collection of Samples:

Sarcocyst muscle cysts were collected from slaughtered buffaloes at different abattoirs of Menofia province, Egypt during the period extended from November 2014 to October 2015. All data about the slaughtered buffaloes were recorded including age, sex, locality and season. Fresh sarcocysts were extracted from muscle tissues with sterile brand needles for each cyst, washed several times with normal saline as previously described according to (Yang et al., 2001a). Each cyst was stored in epindurr tube at 20°C till further use.

DNA Extraction: DNA was extracted from different sarcocysts obtained from slaughtered buffaloes using a commercial DNA extraction kit (Qiagen D Neasy Blood and Tissue extraction kit®) according to manufacturer protocol. DNA was analyzed on 2% agarose gel (Agrose Bioshop Biotechnology, Canada) and DNA concentration was measured using spectrophotometer.

PCR Amplification:

Forward (5’-CGCCCTTTTAGTAGGOTTGT3’) and reverse (5’ TACGAATGCCCCAACTGTC 3’) were used to amplify the Targeted DNA sequence. 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 primer tool. ( promiga)1 μL DNA polymerase (2 U), (promiga) 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.

DNA Sequencing, Sequence Alignment and phylogenic 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 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 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).

3. RESULTS

3.1. Prevalence of Sarcocystis species in buffaloes:

In the present study regarding to macroscopical and microscopical examination of sarcocystis from buffalo’s muscle table (1 and 2) revealed the total infection with macroscopic sarcocystis was (82.92 %), but in animals over 5 years it was 92.81% and in animals under 2 years it was 21.54%. Results in table (3) revealed that the esophagus and pharyngeal muscles were the organ most frequently infected with either macroscopic or microscopic sarcocysts (82.92 %) this means that the esophagus and pharyngeal muscles were the organ most frequently infected with either macroscopic or microscopic sarcocysts (82.92 %) this means that the esophagus and pharyngeal muscles were infected in all infected carcasses, followed by the tongue (16.9%), diaphragm (13.7%), masseter muscle (1.2%), light infestation in different muscles specially in the fore quarters (0.77%) and heavy infestation (0.51%). In the present study regarding to the seasonal variations as for the table (4) there is a little variation of infection with sarcocysts species in buffaloe carcasses, in autumn 81 %, winter 87.6%, spring 83.5% and summer 80.1%.

3.2. Morphological and histological examination:
In the present study Fig. (1 and 2) the macroscopical description of *S. fusiformis* revealed spindle shaped cyst characterized by whitish coloration and measurements ranged from 0.3–2.5 × 0.2-0.7 cm, while histopathological examination revealed thick wall cyst measured from 4.4 - 5.4 μm composed of long striated protrusions in a palisade-like arrangement and the cyst was divided with septa into irregular compartments filled with crescentic shape bradyzoites.

The phylogenetic analysis Fig. (4) of the 18S rRNA sequences showed that 6 examined samples were *S. fusiformis* strains the nucleotide homology were 85%, 95%, 97%, 96%, 91% and 96 %, respectively. Furthermore, the cladogram revealed that this group was more associated with the newly identified *S. silva* in 2 examined samples from the Egyptian water buffaloes and the nucleotide homology were 92%, 91% together with *S. bovini* in 1 of the examined samples with nucleotide homology 99%.

Table (1): The prevalence of macroscopic forms of sarcocystis species from the examined buffaloe carcasses in El-Menofia Governorate from different abattoirs.

<table>
<thead>
<tr>
<th>genus</th>
<th>n. of total examined carcasses</th>
<th>n. of infected carcasses</th>
<th>Total %</th>
</tr>
</thead>
<tbody>
<tr>
<td>male</td>
<td>130</td>
<td>28</td>
<td>21.54</td>
</tr>
<tr>
<td>female</td>
<td>807</td>
<td>749</td>
<td>92.81</td>
</tr>
<tr>
<td>total</td>
<td>937</td>
<td>777</td>
<td>82.92</td>
</tr>
</tbody>
</table>

Table (2): The prevalence of macroscopic forms of sarcocystis species in examined buffaloe carcasses from different abattoirs in El-Menofia Governorate.

<table>
<thead>
<tr>
<th>Abattoirs</th>
<th>n. of examined carcasses</th>
<th>n. of infected carcasses</th>
<th>Total n.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
<td>Total n.</td>
</tr>
<tr>
<td>El-Shohada</td>
<td>57</td>
<td>222</td>
<td>279</td>
</tr>
<tr>
<td>El-Batanon</td>
<td>32</td>
<td>215</td>
<td>247</td>
</tr>
<tr>
<td>Shebin El-kom</td>
<td>41</td>
<td>370</td>
<td>411</td>
</tr>
<tr>
<td>Total</td>
<td>130</td>
<td>807</td>
<td>937</td>
</tr>
</tbody>
</table>

Table (3): The prevalence of macroscopic forms of sarcocystis species in buffaloe carcasses at different organs.

<table>
<thead>
<tr>
<th>Infected organ</th>
<th>Male</th>
<th>Female</th>
<th>Total</th>
<th>Total %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Esophagus and pharyngeal muscle</td>
<td>28</td>
<td>749</td>
<td>777</td>
<td>82.92</td>
</tr>
<tr>
<td>Diaphragm</td>
<td>6</td>
<td>123</td>
<td>129</td>
<td>13.70</td>
</tr>
<tr>
<td>Tongue</td>
<td>4</td>
<td>154</td>
<td>158</td>
<td>16.90</td>
</tr>
<tr>
<td>Masseter muscle</td>
<td>1</td>
<td>10</td>
<td>11</td>
<td>1.20</td>
</tr>
<tr>
<td>Light infestation</td>
<td>0</td>
<td>6</td>
<td>6</td>
<td>0.77</td>
</tr>
<tr>
<td>Heavy infestation</td>
<td>0</td>
<td>4</td>
<td>4</td>
<td>0.51</td>
</tr>
</tbody>
</table>
Table (4): The prevalence of macroscopic forms of sarcocystis species in the examined buffalo carcasses at different seasons.

<table>
<thead>
<tr>
<th>Season</th>
<th>Male</th>
<th>Female</th>
<th>Total n.</th>
<th>% of infected carcasses</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Infected carcasses</td>
<td>Non infected carcasses</td>
<td>Infected carcasses</td>
<td>Non infected carcasses</td>
</tr>
<tr>
<td>Autumn</td>
<td>8</td>
<td>43</td>
<td>254</td>
<td>17</td>
</tr>
<tr>
<td>Winter</td>
<td>5</td>
<td>6</td>
<td>172</td>
<td>19</td>
</tr>
<tr>
<td>Spring</td>
<td>8</td>
<td>27</td>
<td>169</td>
<td>8</td>
</tr>
<tr>
<td>Summer</td>
<td>7</td>
<td>26</td>
<td>154</td>
<td>14</td>
</tr>
<tr>
<td>Total</td>
<td>28</td>
<td>102</td>
<td>749</td>
<td>58</td>
</tr>
</tbody>
</table>

Fig. (1): *S. fusiformis* in the esophagus of water buffaloes.

Fig. (2): (A) cross section of sarcocystis cyst in the tongue muscle showed the cyst filled with crescentic shape bradyzoites (H&E x 20); (B) cross section of sarcocystis cyst in the esophogal muscle showed the cyst filled with crescentic shape bradyzoites (H&E x 20); (C) cross section of sarcocystis cyst in the esophogal muscle showed mature giant cyst with thick external wall and thick trabecular septa surround crescentic shape bradyzoites (H&E x 10) and (D) cross section of sarcocystis cyst in the esophogal muscle showed mature giant cyst with thick trabecular septa surround crescentic shape bradyzoites (H&E x 40). Identification of Sarcocystis species by random amplified Polymorphic DNA - Polymerase chain reaction.

Fig. (3): Electrophoresis of the 18S rRNA gene used for PCR assay for the local tested isolates of *S. fusiformis*. In this study 10 samples were examined by PCR of sarcocystis species based on the 18S rRNA gene sequence, the branch lengths are proportional to the amounts of evolutionary changes. M marker. Lane 1, 2, 3, 4, 5, 6, 8, 9 and 10 sarcocysts yielded target DNA fragments and yielded 18SrRNA gene. Lane 7: Negative

Fig. (4): Cladogram (phylogenetic tree) of sarcocystis species based on the 18S rRNA gene sequence. The accession ns. of sarcocystis species.
KR186119 *S. fusiformis*
KP006409 *S. fusiformis*
KC209733 *S. gigantea*
KP006410 *S. taeniata*
AB661441 *S. fayeri*
GQ251023 *S. truncata*
GQ250970 *S. tarandi*
GQ251017 *S. elongata*
KX643338 *S. silva*
KJ778011 *S. cafferi*
KU247907 *S. buffalonis*
KT901161 *S. hirsuta*
KC508513 *S. mouleti*
GQ250980 *S. rangiferi*
EU327974 *S. miescheriana*
AF017120 *S. cruzi*
L24383 *S. tenella*
GQ8999206 *N. caninum* (*Neospora caninum*)
KT901145 *S. bovini*
4. DISCUSSION

Results of the present work indicated a high prevalence of sarcocystosis among slaughtered buffaloes in the region of El-Menofia, Egypt and this suggests that buffaloes are frequently exposed to infection due to their close relationship with dogs, cats, and even wild animals that act as final hosts for these protozoa, where felids are known to be the definitive hosts of macroscopic sarcocysts, whereas canids are considered to be the final host of microscopic forms (Dubey et al., 1989). This results agree with Nassar (1982) who reported that the incidence of infection with both microscopic and macroscopic cysts among water buffaloes in Cairo was 94.0% and 97.7%, respectively, Mohamed (1988) reported that the incidence of sarcocystis species was 38.65%, 66.66% and 92.08% in male buffaloes under 2 years and female buffaloes over 5 years, respectively, Said (1996) who found an infection rate of 76.8% in Assiut Governorate added that elderly buffaloes were more commonly exposed to infection. Similar results were obtained by Fawaz (1998) who detected an infection rate of 72.6% in examined buffaloes in Qena Governorate, Egypt. Hendawy (2006) found that the highest incidence (93.7%) among water buffaloes aged over 5 years, however the animal aged between 1.5-4 years, (18.4%) of them harboured S. fusiformis cysts, El-Sayed (2010) reported that the prevalence of infection with S. fusiformis was 17.2% in buffaloes 2-3 years old and (68.1%) in those over 5 years old, Abdel-Baky (2011) investigate the prevalence of S. fusiformis in water buffaloes the infection rate was 83% the infected animals 41% of inspected male and female 94.56%, and also agree with Rasha (2013) revealed an infection rate of S. fusiformis 75% and S. hominis 7%.

Similer result have also been recorded in other countries that have similar climatic conditions, such as Perrotin and Bermejo (1978) who reported that the incidence of macroscopic sarcocystic cyst in African buffaloes were 76.3%, Huong et al. (1995) found that the incidence of S. fusiformis was (68%), Xiao et al. (1988) in China who recorded that the prevalence of infection by both macro- and microscopic sarcocystis species in buffaloes was 94% Mohanty et al. (1995) 87% in India and Claveria et al. (2000) in philipine revealed that 65%. On the other hand, other studies reported lower incidence as, Ozer (1988) studied the incidence of macroscopic cysts of S. fusiformis in turkey in esophagus and diaphragm, which was (26.2%), however the microscopic cysts were detected (95.1%), Degloorkar et al. (1993) in India (15.31%) in various organs. Huong (1999) in Vietnam found that S. fusiformis infection were (41%), Latif et al.(1999) in Iraq found that infection with macroscopic forms were (15.6%), Muresan et al. (2004) in Transilvania, (12.68%) and Masoud et al. (2007) in Iran revealed that (20%) of inspected animals were infected with S. fusiformis, Khalifa et al. (2008) who recorded infection with S. fusiformis was 28% by macroscopical examination, Asmaa et al. (2013) reported the infection of buffaloes with S. fusiformis was 25.5%, the results do not agree with those of Moré et al. (2008) who said that the predisposition of bulls to higher rate of sarcocystis infection, because bulls are frequently pastured close to farm buildings, thus with an increased chance for contact with final hosts, Ifeoma-Nancy (2012) found that age, sex and breed did not significantly influence the prevalence of sarcocystis infection.

This result agree with Fayer and Dubey (1986), Huong et al. (1995) found that the incidence of S. fusiformis was (68%) in the esophagus, (45%) in the tongue, (27%) in the cerebral muscles and (5%) in the abdominal muscles, Degloorkar et al.(1993) in India found that incidence was in the tongue (11.37%), musculature (2.80%) and masseter muscle (1.49%) Claveria et al. (2000) in philipine revealed that 65% of the carcasses had sarcocystis and two forms of microscopic sarcocystis and Ozer (1988) studied the incidence of macroscopic cysts of S. fusiformis in turkey in oesophagus and diaphragm, which was (26.2%), However the microscopic cysts were detected (95.1%), Oryan et al. (2010) esophagus, tongue, the diaphragm were found to be the most common sites for sarcocystis species. infection in buffaloes. The results partially agree with Daryani et al. (2006) found that the abdominal muscles of infected buffaloes were more frequently infected than the esophagus. Evidently, distribution of sarcocysts does not follow a specific pattern in most of the infected organs in buffaloes, with the exception of macroscopic cysts, which tend to be located in the esophagus.

This result agrees with Shash (1983) found that the seasonal prevalence of sarcocystis infection in domestic animal’s peaks coincides with rainy season, Abdel-Baky (2011) found that the prevalence of S. fusiformis cysts among water buffaloes revealed a very little variation in infection rate in female water buffaloes which were around 94% while in male buffaloes the percentage
of infection were around 33% except in summer the percentage of infection was 25.71%.

The result revealed the possibility of infection in buffaloes aged 1.5 years and older, we were unable to use animals younger than 1-year-old in the present study, as regulations governing abattoirs in Egypt do not allow slaughtering before this age. Buffaloes are widely infected with sarcocystis species.

The Morphological and histological examination of different Sarcocystis species in buffaloes: These results partially agree with Charles (1966), who described the cells of S. fusiformis cyst and its wall, the external boundary of the cyst wall consisted of alternating long and short villous-like projection. The cells of the cyst contained three regions. The anterior third contained sarconemes, which seemed to originate in the conoids. The middle zone contained mitochondria and many bodies of various sizes, shapes and electron density. The posterior portion of the cell contained apromatic nucleus, osmophilic bodies, and mitochondria. As the same aspect the results agree with Abdel Ghaffar et al. (1978) who recorded the measurement of S. fusiformis after examination of the muscular layer of the esophagus of 20 Indian water buffaloes (7-10 years old). The large type ranged from 7-30 X 3-7 mm and the small cysts measured 1.3-5.1 X 0.7-2.2 mm. The wall of the large and small cysts showed no distinct morphological differences, also results partially agree with Charles (1966), who described the cells of S. fusiformis as macroscopic, fusiform or spindle -shaped cyst. As the same aspect the results agree with Huong (1999) who recorded that S. fusiformis appear as white opaque bodies, similar to rice grains and their size ranged from 3-38mm.

Identification of Sarcocystis species by random amplified Polymorphic DNA - Polymerase chain reaction: Sarcocystosis has been diagnosed by several methods, digestion, trichinoscope, staining with methylene blue and histological techniques. In addition, IFAT, ELISA, Agglutination and hemagglutination methods are used for the serological diagnosis of sarcocystosis. Although serological techniques are available for diagnosis of sarcocystis species, they are not widely accepted since they do not differentiate the species level. Serological methods show significant variability due to unstable reactive used in these techniques. In addition, it is reported that serological methods may lead to speculative results jenum et al. (1998). The unsuitability of serological tests for the diagnosis of infection have been reported to be due to high titres of antibodies in intermediate hosts originating from enzootic stability and from a high prevalence of infection Pereira and Bermejo (1988). Alternatively, species level identification using microscope techniques is possible in chronic periods of infection, however this method is not useful in acute phase of infection or in low-level of infections.

Recently, molecular based techniques have been used to detect sarcocystis species in intermediate hosts. Molecular techniques have become widely accepted in the world. They provide more specific method than conventionally employed in epidemiological studies Williams et al. (1990). 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, (2007b) and Jehle et al. (2009).

The conserved regions of 18S rRNA gene help in designing primers that can be used to amplify the samegene in related species. Its mosaic structure allows flexibility in experimental designs for various phylogenic 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.

In the present study, S. fusiformis primer Forward (5`- CGCCCTTTTAGTGAGGTTG3`) and reverse (5` TACGAATGCCTACACTGTC 3`) were used to amplify the Targeted DNA sequence. In this study examined 10 samples by PCR of sarcocystis species based on the 18S rRNA gene sequence.

The results of the phylogenetic tree analysis Fig. (4). indicated that our local tested isolates of S. fusiformis were closely related to S. fusiformis accession n. (KR186119, KP006409) and this was reflected by the high nucleotide homology (95%, 96%, 96% and 97 %), respectively between them. On the contrary, the relationship with the tested isolate of S. fusiformis was nearly different and represented by (91% and 85%) nucleotide homology, suggesting a distinct genetic difference between the tested isolates of S. fusiformis investigated in the current study. Also the phylogenetic analysis represents a new species S. silva which are closely related to S. silva accession n. KX643338 and another new species. S. bovini in sample which is closely related to S. bovini accession n. KT901145, this result is fully agree with Oryan (2011) stated that a comparison
between the alignments between the Iranian 18 S rDNA sequences (HQ703791), made in this study, and those previously reported for S. fusiformis in different geographical location (accession ns. AF176927, AF176926, and U03071) showed the occurrence of local genetic polymorphisms and heterogeneity in this ribosomal location, El-Seify et al. (2014), who studied water buffaloes and screen the infection rates and apply genetic identification of macroscopic sarcocystis species infecting Egyptian water buffaloes in El-Gharbia Governorate, Egypt. 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, 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 (1 3 positional differences).

In the present study, phylogenetic analysis revealed that sequences of the local tested isolates of S. fusiformis, S. bovini, S. silva shared the same definitive host (cat) and formed one cluster. The 18S rRNA gene is characterized by the presences of various location of polymorphism between the different species of sarcocystis and within same species Holmdahl et al. (1999) and Oryan et al. (2011) this was observed in the current study and indicated by differences in the nucleotide sequences from the same sarcocystis species. Accordingly, the present Egyptian S. fusiformis differed by (3%, 4% and 5%) and (9 and 15%) from those previously recorded from China and Sweden, respectively Yang et al. (2001b) and Holmdahl et al. (1994). Therefore, examination of more sarcocystis from other geographic locations and sequencing at more genetic location (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

The obtained results confirmed that Egyptian domestic buffaloes are widely infected with sarcocystis species this high infection rate may be due to the abundance of final hosts, especially dogs and cats, that encourage the spreading of infection by this protozoan. There is higher infection rate with both macroscopic S. fusiformis within older age water buffaloes were observed. This may be due to that the sarcocysts need longer time to appear macroscopically together with the first record of S. silva and S. bovini in Egyptian buffaloes depending up on the molecular differences between the two isolates as deduced from the analyses of 18S rRNA gene sequences. 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.

6. REFERENCES


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