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Molecular identification of Sarcocystis species in imported frozen beef in Egypt

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Abstract

In the present study, we set out to implement and compare the different detection methods that include the peptide digestion, histopathological examination and PCR, to identify Sarcocystis species that can be found in the imported frozen beef. Comparing results from the three identification methods, along with their compatibility and accuracy in diagnosing Sarcocystis spp. are also presented. Finally, a special focus is given to the use of PCR followed by sequencing and the bioinformatics analysis in detecting and comparing Sarcocystis found in the imported frozen beef. A total number of 384 samples of imported frozen beef (280 Indian buffalo and 104 Brazilian cattle meat) were collected from different retail meat shops in Alexandria province, Egypt. Visual meat inspection revealed a low prevalence of macroscopic Sarcocystis spp. in buffalo beef (3.9%) and no macrocysts were detected in cattle beef. Fifty samples were taken from buffalo beef and subjected to peptic digestion, histopathology and PCR resulting in 24%, 38% and 34% positive samples respectively. While the same number of samples were taken from cattle beef resulting in lower infection rates (6%, 2% and 2%). There were no significance difference found between the infection rate according to diagnostic method or type of meat cut. For molecular characterization newly designed primer sets were used to specifically amplify the 18S DNA sequences of the four Sarcocystis spp. in both buffalo and cattle (S.fusiformis, S.buffalonis, S.hirsuta and S.hominis) using the primer BLAST tool on the NCBI of the Genbank. Aligning the 18S rDNA sequences of three positive PCR products revealed high identity percents with three Sarcocystis spp.; S. fusiformis, S. buffalonis and S. hominis. (85%, 92% and 100% respectively).

1. INTRODUCTION

Species of Sarcocystis (Apicomplexa: Sarcocystidae) are coccidian parasites of warm blooded and poikilothermic animals, including humans. More than 200 species of Sarcocystis are recognized however, complete life cycles are known only for 26 species. Sarcocystis spp. usually have a two-host, prey-predator life cycle pattern, with herbivores as intermediate hosts and carnivores as definitive hosts (Dubey et al. 2015a). Four characterized species of Sarcocystis have been reported and described in the water buffalo (Bubalus bubalus) as an intermediate host. Sarcocystis fusiformis and Sarcocystis buffalonis form macroscopic Sarcocysts with cats as the definitive hosts, while Sarcocystis levinei and Sarcocystis dubeyi form microscopic Sarcocysts. Despite dogs are reported as the definitive hosts for S. levinei, the definitive host (s) for S. dubeyi has not been identified yet (Hilali et al. 2011). Cattle (Bos taurus) are intermediate hosts for five species of Sarcocystis, namely S. cruzi, S. hirsuta, S. hominis, S. rommeli and S. heydorni. Of these species, only S. hominis and S. heydorni are zoonotic (Dubey et al. 2015b). Few reports were conducted on the presence of Sarcocystis spp. in the imported frozen beef (Radwan et al. 2015; El-Kelesh et al. 2011), in particular in the frozen Indian and Brazilian meat, which are the most common import sources of frozen meat in Egypt (Al-Habbal, 2015). Of much concern in particular is the potential presence of

microscopic Sarcocystis spp., which are most likely to go undetected with the traditional examination method of visual inspection .

The present study was carried out to investigate the prevalence of Sarcocystis spp. in the imported frozen Indian buffalo and Brazilian cattle beef in Alexandria province, Egypt. A special focus is given to the use of PCR followed by sequencing and the bioinformatics analysis in detecting and comparing Sarcocystis spp. found in the imported frozen beef

2. MATERIAL AND METHODS

2.1. Collection of samples

Specimens were obtained from 384 imported frozen beef (280 Indian buffalo and 104 Brazilian cattle meat) from different butcheries, supermarkets and retail meat shops in Alexandria province, Egypt, during the period from November 2014 to March 2016. Each sample weighted at least 200 g from different beef cuts (forequarters and hindquarters). Samples were kept inside an icebox with ice bags and sent immediately to the Department of Parasitology, Faculty of Veterinary Medicine, Alexandria University, to be examined by visual inspection. After that, hierarchical selection of samples (50 for each buffalo and cattle beef) for subsequent examinations (digestion, histopathology and PCR) was carried out. Samples were kept at -20 ºC until microscopic examination and DNA extraction for molecular studies.

2.2. Gross examination (Visual inspection)

For the visual inspection, frozen samples were left to be thawed for about 2 hours before examination. All muscle samples were examined grossly for the presence of Sarcocystis spp. macrocysts. Muscle cuts were then sliced to facilitate the visual inspection of meat slices to detect any cysts. Macroscopicaly visible cysts were carefully isolated from samples and then stored in 70 % ethanol .

2.3. Peptic digestion

Digestion of muscle samples was carried out according to the method described by Dubey et al. (1989) with some modifications. Briefly, a 10 g obtained from each sample, cut into small pieces and then transferred to a glass beaker containing 50 ml of the peptic digestion solution with the following composition: 1.3 g pepsin (LOBAL Chemie, India; 1:10,000), 2.5 g NaCl, and 3.5 ml of HCl, made up to 500 ml with distilled water. The digestion process was carried out in an incubator at 40 °C for 20 minutes for digestion of muscle fibers .

.2.4Histopathological examination

Tissue samples were fixed in 10% neutral-buffered formalin and processed for standard histological technique according to Bancroft and Gamble (2002). Slides stained with haematoxylin and eosin (H&E) were examined under the light microscope for detection of microscopic Sarcocystis. Histopathological preparation was carried out in the Department of Pathology of the Public Health High Institute, Alexandria University.

2.5. Molecular assays

2.5.1. DNA Extraction

Total genomic DNA was extracted from muscle tissue samples using the Qiagen DNeasy Blood and Tissue kit (Qiagen®; Germany) according to the manufacturer protocol. Genomic DNA extracts were then stored at -20 °C .

2.5.2. Agarose gel electrophoresis

Extracted DNA samples were elecrophoresed through 1.5% agarose solution in 1x TBE electrophoresis buffer at 80 V for 100 minutes, according to the method described by Daptardarkar et al. (2016) with slight modifications. After staining in ethidium bromide for 30 minutes, DNA banding patterns were acquired by the gel documentation system (Photodoc-it, UVP, England), and data were analyzed using the Totallab analysis software (Ver.1.0.1).

2.5.3. PCR Amplification

Four sets of primer pair were designed to amplify targets of Sarcocystis spp. These primers were designed to specifically amplify targets from the 18S rRNA sequences, and were named from 1 to 4, with the accession numbers of U03071, AF017121, JX855283, JX679471, respectively (Table 1). Primers were designed by using the primer BLAST tool of the NCBI Genbank (http://www.ncbi.nlm.nih.gov/tools/primerblast/primertool.cgi). First, PCR reactions were set for buffalo and cattle beef samples by using the the first primer set (Primer 1), which is specific for four Sarcocystis spp. namely as S. fusiformis, S. buffalonis, S. hirsuta and S. hominis) (Table 1). In PCR tubes, PCR reactions were set up containing the PCR master mix (DreamTaq Green PCR Master Mix; ThermoScientific®) of buffer, deoxynucleotides, DNA polymerase. To each PCR reaction, a 5µl of the genomic DNA (contain ~25 ng) and 10 µm (1.0 µl) of each primer was added. The PCR amplification reactions were carried out in the Thermocycler (TECHNE/ TC 3000-Bar world

scientific, UK). PCR reaction conditions were as follow: initial denaturation for 2 min at 94 °C; 35 cycles with denaturation at 94 °C for 30 sec, annealing at 52 °C for 30 sec and extension at 72°C for 30 sec; and final extension for10 min at 72 °C. PCR products were finally analyzed by the agarose gel electrophoresis (as mentioned before), banding patterns were documented, and the positive PCR products were stored at –20 °C till used for the corresponding DNA sequencing .

As the designed primer 1 is conserved and shared between four Sarcocystis spp., all positive PCR products with primer 1 were subjected to subsequent PCR with the more specific primers (Primer 2, 3, 4) (Table 1). A similar PCR amplification and detection protocol was followed as above with exception of minor modifications; initial denaturation for 2 min at 94 °C; 40 cycles with denaturation at 94 °C for 30 sec, annealing at 50 °C for 30 sec and extension at 72°C for 30 sec. and final extension for 10 min at 72 °C.

Table (1): List of newly designed primers used for PCR and sequencing of 18S rRNA gene of Sarcocystis spp.

	Primers	Sequence 5′→3′	Amplicon Size (bp)	*Sarcocystis* Species
1	Forward	CGAATGGCTCATTAAAACAG	570	Common in 4 Spp. (S.fusiformis, S.buffalonis, S.hirsuta & S.hominis)
	Reverse	CCAACTACGAGCTTTTTAAC		
2	Forward	CGCAAATACTATATCACTCG	689	Specific for S.fusiformis
	Reverse	CTAGAAACCAACAAAATAGA		
3	Forward	ATGTCACTTTACGGTGGATG	490	Specific for S.buffalonis
	Reverse	GTACACACCCTCACTAAAAGG		
4	Forward	ATTATTATGATTGGCGATAG	554	Specific for S.hominis
	Reverse	CTACAAACCAACAAAATAAT		

2.5.4. DNA Sequencing

Three representative samples from positive PCR products were selected for DNA sequencing in one direction using the forward primer (the same used in PCR amplification with primer 1). Sequencing reactions and protocols were performed according to manufacturer procedure using the ABI PRISM® 3100 Genetic Analyzer (Micron-Corp. Korea). Resultant DNA nucleotide sequences were then subjected to the BLAST and reverse-BLAST analysis at NCBI (Altschul et al. 1990).

2.5.5. hylogenetic (Maximum Parsimony) analysis of molecular data

The evolutionary history was inferred using the Maximum Parsimony method. The most parsimonious tree with length = 527 is shown. The consistency index is (0.753813), the retention index is (0.819489), and the composite index is 0.643773 (0.617741) for all sites and parsimony-informative sites (in parentheses). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm (Nei and Kumar, 2000) with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates .(

2.6. Statistical analysis:

According to method described by Kotz et al. (2006) and Kirkpatrick & Feeney (2013), data were fed to a computer and analyzed using IBM SPSS software package version 20.0. Qualitative data were described using number and percent. Significance of the obtained results was judged at the 5% level. (The minimum level of significance was set at $p \leq 0.05$).

3. RESULTS

3.1. Visual inspection

As shown in Table 2, eleven out of 280 of imported frozen buffalo beef samples (3.9%) were found infested with visible Sarcocystis spp. Macroscopic cysts were opaque white in color, spindle shaped, measured 0.5-6.0×0.2-0.5 cm and located sometimes just beneath the serosal surface or deeply situated within the skeletal musculature (Fig. 1). No gross lesions were found in the affected muscle tissues. On the other hand, no macroscopic visible cysts were detected in the cattle beef samples. However, there was no significant difference between positive samples infected with macroscopic Sarcocystis in each type of meat cut of buffalo beef and that of cattle beef (Table 2).

3.2. Microscopic detection

Higher rates of infestation is detected by the peptic digestion method, with 24% (12/50) of buffalo beef and 6% (3/50) of cattle beef, respectively, are found with detectable stages of Sarcocystis as individual bradyzoites (Fig. 1). Histopathological examination also turned up with higher rates of Sarcocystis spp. cysts infestation with buffalo beef with an incidence of 38% (19/50) while in cattle beef it was 2% (1/50). Careful inspection of histopathology results also reveal Sarcocystis with variable morphologies and sizes, with the majority of cysts are globular to oval-shape (Fig. 2). When categorizing cysts based on cyst wall thickness, 68.4% (13/19) of detectable microcysts in buffalo beef are thick walled, while minorities 6/19 (31%) are thin walled microcysts (Fig. 2). Most of the thick cyst walls 61.5% (8/13) are transversely striated with conical villar protrusions on its surface while only 38.4% (5/13) have no protrusions. On the other hand, thin cyst wall has smooth surface with no structures on it (Fig. 2). Also in the general theme of low prevalence, only a single cattle beef sample is found to possess a thick walled microcyst after the histopathology examination (Fig. 2). Microscopic Sarcocystis were measured using Otify (2012) method. From the histology perspectives, the infected muscle fibers are swollen showing loss of striation and fragmentation with interstitial edema and aggregation of inflammatory cells.

Table (2): Sarcocystis spp. infection in the examined imported frozen beef (different meat cuts) by visual examination.

Item / Meat cut	No. of visually examined samples		Positive samples by visual inspection				^{FE}p
			Buffalo		Cattle		
	Buffalo	Cattle	No.	%	No.	%	
Neck	41	16	4	1.4	0	0	0.568
Chuck	78	21	2	0.7	0	0	1.000
Shoulder	68	23	2	0.7	0	0	1.000
Knuckle	31	19	1	0.3	0	0	1.000
Topside	35	15	1	0.3	0	0	1.000
Rump	27	10	1	0.3	0	0	1.000
Total	280	104	11	3.9	0	0	

*: Statistically significant at $p \leq 0.05$

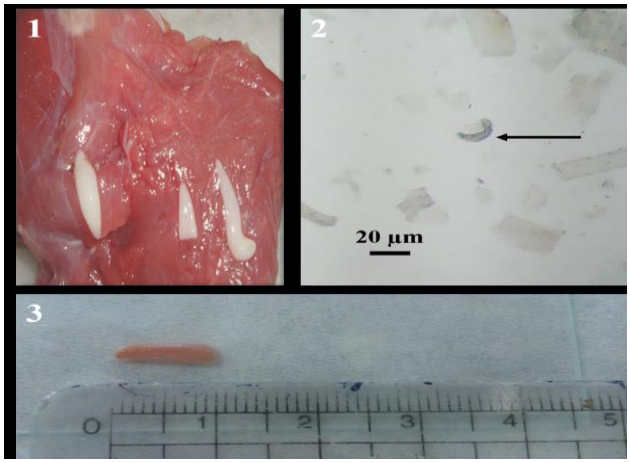

Fig. 1.

1) Macroscopic *Sarcocystis* spp. cysts detected in the neck of imported frozen buffalo beef.

2) Banana-shaped bradyzoite (Arrow) under microscope (X40) after peptic digestion of imported frozen buffalo beef.

3) Cysts found in chuck of imported frozen buffalo beef.

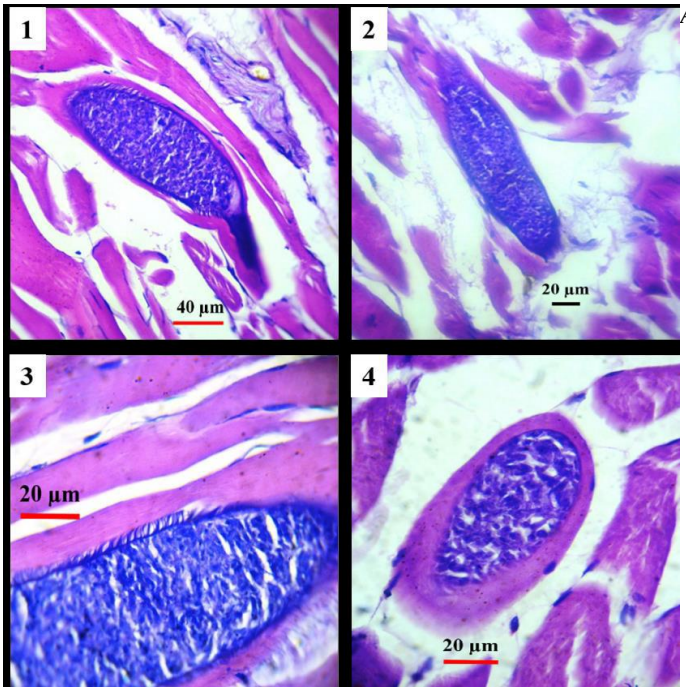

Fig. 2. *Sarcocystis* spp. in histological section with H &E.

1) Thick walled cyst in buffalo meat.

2) Thin walled cyst in buffalo meat.

3) Thick walled cyst with fine protrusion on its surface in buffalo.

4) Thick walled cyst in cattle meat.

3.3. Molecular identification

Seventeen out of fifty buffalo samples (34%) were found positive by PCR using the conserved common primer, while only one cattle sample is positive (2%), with the PCR product of expected molecular mass of 570 bp (Fig. 3). However, there was no significant difference between the results of three examination methods used to detect Sarcocystis infection in the examined imported frozen beef (Table 3). As PCR with a common primer cannot distinguish between individual Sarcocystis species, PCR reactions with more specific primers carried out on representative samples with positive common PCR products. Of these, positive PCR products are obtained with molecular masses of 689, 490 and 554 bp; that correspond to the 18S rRNA sequence targets from S. fusiformis, S. buffalonis and S. hominis, respectively (Fig. 4).

Assemblage of sequencing data performed on three representative samples, macroscopic Sarcocyst from buffalo's neck, microscopic Sarcocyst from buffalo's chuck and microscopic Sarcocyst from cattle's chuck, in one direction using the forward primer yielded three sequences of 560 bp, 565 bp and 566, respectively. Based on the BLAST sequence comparative analysis, the three chosen samples can be easily aligned to three published Sarcocyst isolates, based on their identity and similarity scores (Table 4).

The similarity percent of the Indian macroscopic Sarcocystis isolate in this study with the published data of S. fusiformis under Genbank accession number KR186135.1: (Gjerde et al. 2015) were 83.8%. In addition, the similarities between the current Indian microcyst and the published data of S. buffalonis under accession number KU247925.1: (Gjerde et al. 2016) were 67.4%. The sequence identity between our Brazilian microscopic Sarcocystis isolate and the published data of S.hominis (accession number KP006407.1 Nader & Ghoneim (2014) was 68.3%. Finally, the partial 18S rRNA gene sequences of our imported Indian isolates of macroscopic and microscopic Sarcocystis spp. showed 2.1% dissimilarity.

Phylogenetic analysis of the partial 18S rDNA nucleotide sequences of Sarcocystis spp. using maximum parsimony (MP) (Fig.3) showed that the Indian isolate of macrocyst (from the present study) placed in a group with the three previously reported Sarcocystis spp. (S. fusiformis, S. buffalonis and S. hirsuta) with accession numbers KR186135, KU247925 and KT901220 respectively. In addition, the Indian microcyst isolate (from the present study) formed a cluster with S. cafferi, S. cruzi and S. masoni with accession numbers KJ778019, KT964025 and KU527113 respectively. Furthermore, the cladogram revealed that the Brazilian isolate of microcyst was more associated with S. hominis (accession number KP006407.1) (Fig.5).

Table (3): *Sarcocystis* spp. infection in the examined imported frozen beef by different examination methods.

Item / Origin	No. of examined samples	No. of Positive samples detected by			p
		Peptic Digestion	Histopathological Examination	PCR	
Buffalo	50	12	19	17	0.303
Cattle	50	3	1	1	$^{MC}p=0.619$
Total	100	15	20	18	

Table (4): Blast analysis of three positive PCR products with Primer 1 (Sample 1: macroscopic *Sarcocyst* from buffalo's neck, Sample 2: microscopic *Sarcocyst* from buffalo's chuck and Sample 3: microscopic *Sarcocyst* from cattle's chuck).

No.	Accession No.	Blast Identity	Similarity	Description	Reference
Sample 1	KR186135.1	85%	83.8%	*Sarcocystis fusiformis* isolate Bb1.5 clone 1 18S ribosomal RNA gene	Gjerde et al. (2015)
Sample 2	KU247925.1	92%	67.4%	*Sarcocystis buffalonis* isolate Bb18.1 clone 1 18S ribosomal RNA gene	Gjerde et al. (2016)
Sample 3	KP006407.1	100%	68.3%	*Sarcocystis hominis* isolate 140109-20_G08_5 18S ribosomal RNA gene	Nader & Ghoneim (2014)

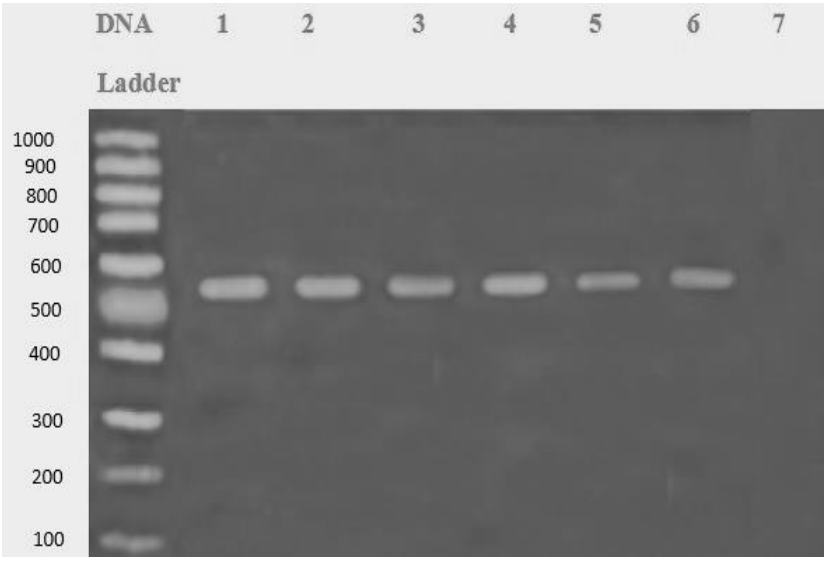

Fig. 3. PCR identification of the 18S rRNA gene of *Sarcocystis spp*. with primer 1. Lane (M 100bp) DNA ladder. Lanes 1-5, the positive diagnostic bands of the tested buffalo specimens. Lane 6 is the only positive band of the tested cattle specimens (approx. 570 bp.). Lane 7, negative control.

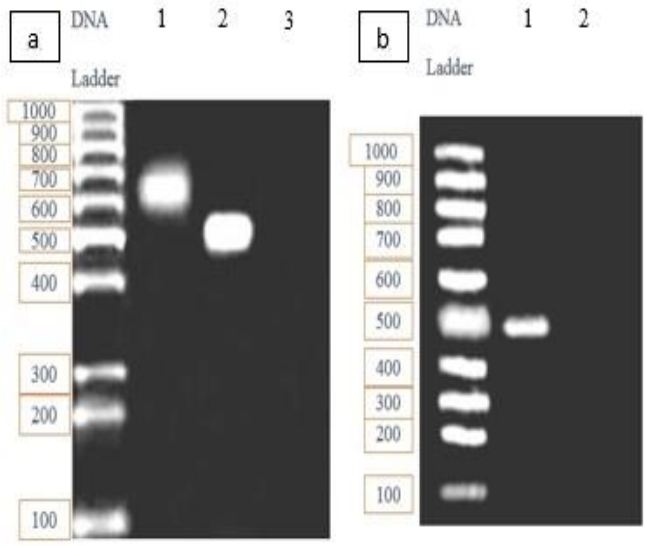

Fig. 4. PCR identification of the 18S rRNA gene for the sequenced isolates of *Sarcocystis* spp. 1000-bp ladder;

1. Lanes 1 & 2, the positive diagnostic bands of the macroscopic *Sarcocystis* from buffalo's neck with primer 2 and microscopic *Sarcocystis* from cattle's chuck with primer 4, respectively (approx. 689 & 554 bp.). Lane 3, negative control.

2. Lane 1, positive diagnostic bands of the microscopic *Sarcocystis* from buffalo's chuck with primer 3 (approx. 490 bp.). Lane 2, negative control.

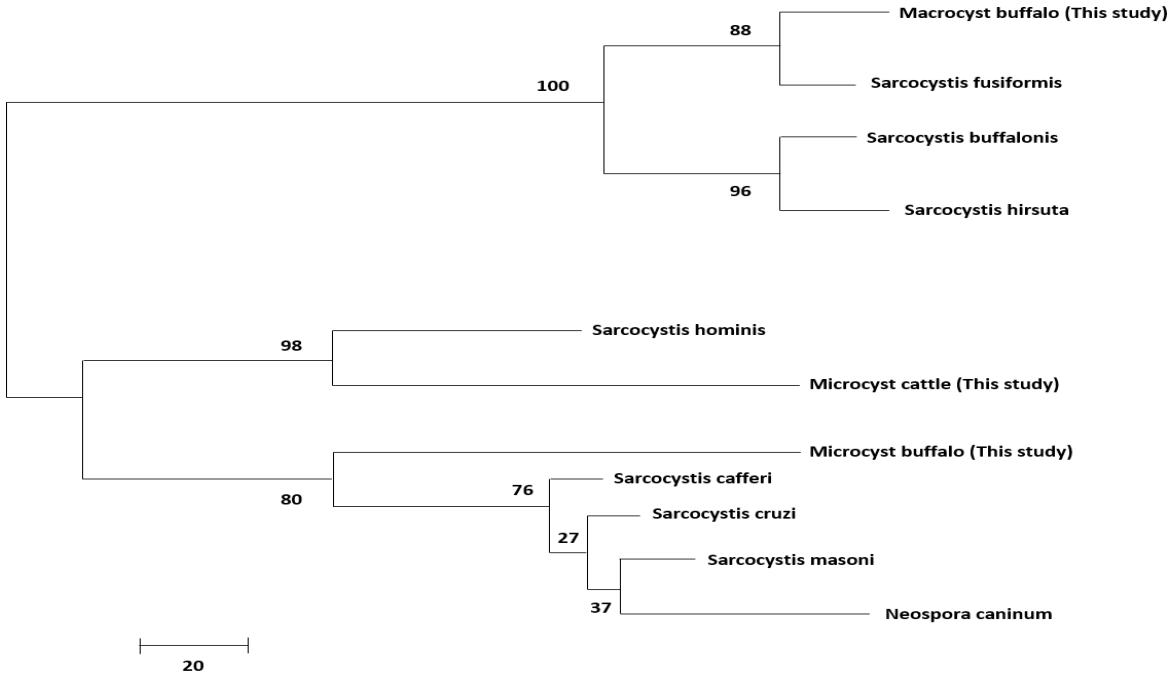

Fig.5. Maximum parsimony (MP) phylogenetic tree was built by alignment of partial 18S rDNA nucleotide sequences with that from other Sarcocystis species deposited in the Genbank. Values on tree nodes indicate bootstrap percentages as calculated from 1,000 bootstrap replicates by using the ClustalW alignment model implemented by MEGA software version 7.0 (Kumar et al., 2016). As a tree outgroup, partial 18S rDNA sequence from Neospora caninum was employed as a distant species. The scale bar indicates branches distance.

4. DISCUSSION

Only few reports investigated the prevalence of microscopic Sarcocystis spp. in the imported frozen beef in Egypt using molecular assays. This may be due to the common concept that both freezing and cooking reduce the infectivity of Sarcocystis (Fayer, 1975). In addition, the skeletal muscles are not severely affected by Sarcocystis and its prevalence is relatively low, although these muscles are the most often eaten parts of beef (Ono & Ohsumi, 1999). However, many studies investigated the presence of Sarcocystis spp. in frozen tissues (Ono

& Ohsumi, 1999; Chen et al, 2007; Radwan et al. 2015).

The current study was carried out on the Indian and Brazilian beef meat as they represent the most common import sources of frozen meat in Egypt (Al-Habbal, 2015). Many researchers in India have reported Sarcocystosis in cattle (Chhabra & Samantaray, 2013; Kalita et al. 2015). Recently, rejection of beef from Brazil and Argentina due to Sarcocystis spp. infection was reported in the last 5 years by the rapid alert system for food and feed (RASFF) adopted by the European Community (Moré et al. 2011).

By visual inspection, only 11 buffalo samples out of 280 were positive to macroscopic Sarcocystis spp. representing low infection rate (3.9%). It seems that the main reason for lack of macrocyst of Sarcocysts is related to the observation and condemnation of macrocysts by the meat inspectors during official inspection in the slaughterhouse of the importing country. This result is nearly as low as that recorded by El-Dakhly et al. (2011) in Egypt (6.9%). On the other hand, other studies revealed high incidence of macroscopic Sarcocystis in water buffaloes, probably because this was carried out on slaughtered fresh meat, as 67.6 % in Egypt (Ashmawy et al. 2014) and 88% in Vietnam (Huong et al. 1995),. There were no macrocysts detected in cattle beef in our study and this agrees with Hamidinejat et al. (2015) who found no macrocyst in cattle.

Different diagnostic methods used in the present study (peptic digestion, histopathology and PCR), revealed a low prevalence of Sarcocystis infection among the imported frozen buffalo beef (24%, 38% and 34% positive samples respectively). These results agrees with Mousa et al. (2015) who recorded only 32.7% as positive infection for Sarcocystis in Indian frozen buffalo meat .

However, there have been reports indicating higher prevalence of Sarcocystis but in fresh buffalo meat as in India (87%) Mohanty et al. (1995), 82.9% in Iraq (Latif et al. 1999), 79% in Vietnam (Huong 1999) and 78.9% in Egypt (El-Dakhly et al. 2011). Low infection rate in Brazilian cattle beef in this study (6%) disagrees with Mowafy et al. (2003) who found 100% of cattle muscle samples were infected in Egypt. While the current research agrees with Ghisleni et al., (2006) who reported that the density for South American beef, especially Brazilian beef, was very mild compared with others. Moreover, Mousa et al. (2015) recorded no Sarcocystis infection among the imported frozen cattle beef .

Concerning the peptic digestion method, the lowest rate of Sarcocystis infection was obtained (24%) in buffalo samples. This may be due to the low intensity of Sarcocystis in each sample (200 g). This result agrees with Chen et al. (2007) who showed that as frozen storage time's increase, bradyzoite structure becomes indistinct; their arrangement becomes disordered and only electron dense bodies and amylopectin can be seen. In addition, the bounds of the plasma lemma become indistinct, the inner membrane complex, microneme and nucleus of the bradyzoites degenerate and the bradyzoites themselves even dissolve. Moreover, Savini et al. (1994) reported that peptic digestion is not considered the most appropriate method for large-scale monitoring programs because it is costly and time-consuming .

Our histopathological studies revealed a large number of thick wall microcysts in buffalo beef that may to be S. dubeyi depending on morphological criteria, and less common thin wall microcysts (S.levinei or S.cruzi). All cattle samples were negative to Sarcocystis except one sample was positive with thick wall microcysts, which appeared to be S. hirsuta or S. hominis. On the other hand, Dafedar et al. (2011) recorded large number of thin wall microcysts of S. cruzi in Indian cattle. Our current study agrees with Ghisleni et al. (2006) who mentioned that the histological differentiation between bovine thin wall Sarcocystis (S. cruzi) from thick wall (S. hirsuta and S. hominis) is simple, whereas the distinction between S. hirsuta and S. hominis is difficult. . In addition, Dubey (2015) recognized several Sarcocysts spp. based on the structure of the cyst walls .

Evidently, distribution of Sarcocystis spp. 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 (El-Dakhly et al. 2011). However, Sarcocystis are mostly found in predilection seats other than muscles as esophagus, tongue and diaphragm (Dafedar et al. 2011; Abu-Elwafa et al. 2015). These organs were not included in the current study, as they are not imported in Egypt. According to the type of meat cut, the incidence of Sarcocystis infection in forequarter beef in this study (48%) was higher than that of hindquarters (22%). Chuck had the highest Sarcocystis infection rate in buffalo beef, 18% (9/50), than other types of meat cuts .

Our results agree with Huong (1999) who found S.fusiformis in skeletal muscles, mainly abdominal muscles (49%), cervical muscles (44%), and reported that S.buffalonis were present in cervical muscles (14.3%), abdominal muscles (16%). In addition, Oryan et al. (2010) recorded very low infection rate of Sarcocystis in thigh muscles (less than 3%). Moreover, Abu-Elwafa et al. (2015) recorded high prevalence of Sarcocystis in throat muscles 56.35% (182/323 infected animal). While Radwan et al. (2015) recorded high infection rate in shoulder (81.2%).

Since the appearance of Sarcocystis is quite variable depending on their location and developmental stage, the molecular studies have been suggested to confirm the morphologically identified species (Kia et al. 2011). Various molecular techniques such as PCR have been used regarding the sensitivity and rapidity to detect Sarcocystis spp. in intermediate hosts (Pritt et al. 2008) and subsequent sequencing of isolated rRNA to determine the genetic diversity among many parasites, phylogenetic and taxonomic studies and in epidemiological mapping (Maurer 2011). 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 analysis (El-Seify et al. 2014). Therefore, it is a valuable to identify Sarcocystis species based on analysis of this gene.

In the present study, we found that molecular methods can be applied for the diagnosis of Sarcocystis spp. in frozen tissues and this agrees with Chen et al (2007) who proposed that freezing is a convenient storage method for samples used in taxonomic studies of Sarcocystis. Moreover, Sarcocystis cyst in muscle could be stored at - 20 °C and remain suitable for ultrastructural morphological study .

The results of phylogenetic analysis revealed that there was a far relationship between the imported Indian macroscopic and the Brazilian tested isolates according to the achieved sequences of 18S rRNA gene. Meanwhile, the cladogram indicated that our Indian macroscopic and microscopic Sarcocystis isolates were closely related to S. fusiformis, accession number KR186135.1; (Gjerde et al. 2015) and S. cafferi, accession number KJ778019; (Dubey et al. 2014), respectively and this was reflected by the high nucleotide homology (83.8%,67.7%) between them. However, the phylogenetic analysis of Daptardarkar et al (2016) indicated that more than one Sarcocystis species are circulating in cattle and water buffaloes in India .

Phylogenetic analysis revealed that sequences of the imported Indian macroscopic Sarcocystis isolate, S. fusiformis (KR186135), S. buffalonis (KU247925) and S. hirsuta (KT901220) shared the same definitive host (cat) and formed one cluster. Moreover, Indian microscopic isolate showed only 3.7% dissimilarity with S. cafferi (KJ778019) and this agrees with Gjerde et al. (2015) who mentioned that only the mitochondrial cytochrome c oxidase subunit I (cox 1) sequences were able to clearly separate the water buffalo Sarcocystis spp. from the morphologically similar species (S. cafferi) in the African buffalo. The current Indian microscopic Sarcocyst isolate differed by (3.5 %) and (3.6 %) from S. buffalonis and S. hirsuta those previously recorded from Egypt and Argentina, respectively (Gjerde et al. 2016).

Another possibility of nucleotide variability within the same Sarcocystis spp. is that, water buffaloes in different geographic regions may harbor different strains of the parasite (El-Seify et al. 2014). Therefore, examination of more Sarcocysts from other geographic locations and sequencing at more genetic loci may elucidate whether there are more differences within the same species. More efforts in sanitary education and in excluding dogs from animal houses/facilities are needed.

5. CONCLUSION

As there were no significance difference between the types of diagnostic methods used in this study, PCR could be applied as a routine laboratory examination for the diagnosis of Sarcocystis spp. in the imported frozen buffalo and cattle beef using the newly designed primers. Further studies should be carried out on prevalence of Sarcocystis spp. in other imported frozen organs as heart.

8. REFERENCES

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