

## Characterization of Sarcocystis Species Based on Traditional and Molecular Methods in Imported Frozen Buffalo Meat in Egypt.

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### Abstract

#### Key words:

Sarcocystis, frozen buffalo meat, PCR, 18S rRNA

Sarcocystis is an intracellular, cyst forming and worldwide distributed parasites. In the present study, fifty five different cuts of imported frozen buffalo meat were collected from different localities of Alexandria markets and were subjected to macroscopic and microscopic examination followed by molecular methods. Polymerase chain reaction used to amplify 18S rRNA gene from the collected samples followed by sequence analysis to detect infections with different Sarcocystis spp. The result revealed that the infection rate in examined sample was 70.9% by molecular methods; while it was 23% and 20% by macroscopic and microscopic examination respectively. Also the percentage of infection was varied among different examined cuts. Comparison of the obtained sequences with published data at the Genbank showed that frozen buffalo meat has multiple sarcocystis infections with *S. fusiformis* and *S. cruzi*. This study supported previous findings indicated that water buffalo could be infected with *S. cruzi* which produced microscopic cyst that has hygienic importance for both veterinary and medical aspects. SO, we recommended to used molecular method for detection sarcocystis infection especially in imported frozen meat to avoid human infection of such zoonotic parasite.

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### 1. INTRODUCTION

The importation of food of animal origin as frozen meat was increased in Egypt especially from India and Brazil (Food and Agricultural Organization, 2009). Inspection of frozen meat play an important role in controlling of number of zoonotic diseases, especially parasites which have effect on meat quality and can be transmitted to man through the consumption of frozen meat. Protozoan of the genus Sarcocystis (*phylum Apicomplexa*), is one of the most common parasites affecting animals. *Sarcocystis spp.* are obligate two-host parasites, The infection of intermediate host (herbivores or omnivores) occur by ingestion of sporulated oocysts or sporocysts The prevalence rate of Sarcocystis is higher in buffalo meat than in cattle (Chhabra and Samantaray 2012).

In water buffalo as intermediate host, four species of sarcocystis have been reported; *S. fusiformis*, *S. buffalonis* as macroscopic sarcocystis (Dubey et al. 1989; Huong et al. 1997), *S. levinei* and *S. dubeyi* as microscopic sarcocystis (Huong and Ussla 1999; Claveria et al. 2000). There is debate whether the Sarcocystis species infesting cattle and buffaloes are the same. This subject is of current

interest because some sarcocystis species as *S. hominis* is proven to be zoonotic in cattle but not in the buffalo. Additionally, in India an experiment indicated that *S. cruzi* of cattle is not transmissible to buffalo (Jain and Shah 1985).

The variable regions of the 18S rRNA gene have been shown to be good genetic markers for characterized certain species of *Sarcocystis* (Yang and Zuo 2000). Pritt et al. (2008) reported that, using molecular method (PCR) may allow greater detection of *Sarcocystis species* in beef than using histological section only. The aim of this work is to study the prevalence of sarcocystis infection in imported frozen buffalo meat by using macroscopic examination, microscopical examination and molecular identification using PCR and sequencing 18S rRNA gene.

### 2. MATERIAL AND METHODS

#### 2.1. Samples collection

A total number of 55 different cuts (Fore quarter (Shoulder-Chuck) and Hind quarter (Silver Side- Knuckle) of frozen boneless Indian buffalo meat were collected from different localities of

Alexandria markets. Each sample was weighted 250 gm and transferred on ice to the Laboratory within 1hr, than kept at -20°C until further examination.

## 2.2. Gross and macroscopic examination

During examination, the meat was thawed and then examined. The samples were sliced by sharp scalpel into very fine slices and thoroughly examined by the naked eye, searching for presence of macroscopic Sarcocystis cysts by using magnifying lens.

## 2.3. Microscopical Examination

Part from collected muscle samples were fixed in 10% formalin and routinely histological examination was performed on paraffin- embedded sections (5µm) stained with hematoxyline and eosin (H&E) according to Bancroft and Stevens (1990). The stained section was examined at magnification (40x) under light microscope and Photos were taken by Digital Camera.

## 2.4. Molecular Identification

### 2.4.1. DNA Extraction and PCR amplification

Prior to molecular identification, the frozen muscle tissue samples and isolated macroscopic sarcocystis cyst were excised from the tissue and preserved in 70 % ethanol at -20°C. The 10 or 30 mg of the ethanol preserved samples were cut into small pieces and digestion in 2mg/ml proteinase k in 700ul of lysis buffer (10mM Tris-HCl (pH 7.5), 10mM EDTA, 50mM NaCl, 2% sodium dodecyl sulphate and 20mM dithiothreitol) .Genomic DNA was extracted for each sample using Phenol-Chloroform extraction according to Sambrook et al. (2001). The extracted DNA was kept at -20°C until further used.

Polymerase Chain Reaction (PCR) was used to amplify partial sequence of the mitochondrial small subunit ribosomal RNA (18S rRNA) gene of sarcocystis genus species by first primer, 18S9LF: 5' GGATAACCTGGTAATTCTATG 3' and 18S1HR: 5' GGCAAATGCTTTCGCAGTAG 3' which used according to Li et al. (2002), this primer amplify fragment of approx. 900 bp and doesn't amplify host DNA. The second primer (F: 5'GGCCCTTTTAGTGAGGGTGT3' and R: 5' TACGAATGCCCAACTGTC 3') used to amplify 18S r RNA sequence (approx. 270 bp) of *S. fusiformis*. PCR was performed for each primer in 50 µL reaction volume, containing 3µL genomic

DNA, 5µl of 10x Dream Taq Green buffer (Thermo Scientific, Germany) 1µl (10 mM)of dNTPs mix (Thermo Scientific, Germany), 1µl of each primer (10 Pmol), 0.6µl Dream Tag DNA polymerase (Thermo Scientific, Germany) and 35 µL dH<sub>2</sub>O which finally added. The reactions were carried out in a thermal cycler (Technee, TC-3000, USA) and thermal cycling program as follows: initial denaturation at 95°C for 5 min followed by 35 cycles of 94°C for 1 min for DNA denaturation, annealing temperature was 56 °C for first primer and 55°C for second for 1 min and extension at 72°C for 1 min and final extension at 72°C for 10 min. The amplified PCR products and GeneRuler 100bp DNA Ladder (Thermo Scientific, Germany) were electrophoresed on 2% agarose gel stained with ethidium bromide. Product band was visualized on a UV Transilluminator and photographed by Gel Documentation system (InGenius, Syngene Bio Imaging, USA).

### 2.4.2. DNA Sequencing and Sequence Alignment

The PCR products of first primer from tissue samples and of second primer from isolated cyst and tissue samples were purified using PCR purification kit (Jena Bioscience, Germany) according to manufacturer instruction. The purified PCR products were sequenced in an ABI 3730XL DNA sequencer (Genetic Analyser, Applied Biosystem, Hitachi, Japan) by using the same primer (forward direction 5`-3`) used in PCR. The results were analyzed using Chromas 1.45 (<http://www.techneleysium.com.au>). The comparison of obtained sequences with those previously published in the NCBI was performed using the BLAST program (<http://www.blast.ncbi.nlm.nih.gov/>) on the basis of highly similar sequences available on Genbank. The sequences were aligned using CLUSTALW version 1.8 (Thompson et al. 1994).

## 3. RESULTS

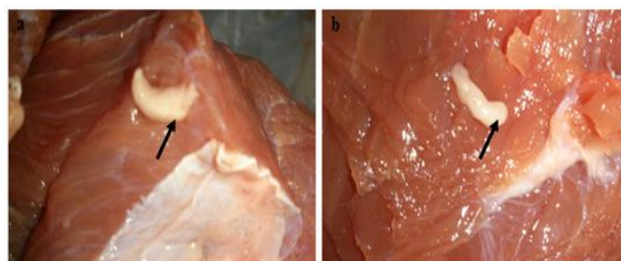
The result of examination of 55 samples of frozen buffalo meat revealed that the sarcocystis infection was positive for both macroscopic and microscopic sarcocystis spp. Where, percentage of positive samples by macroscopical examination methods was 23.6% and 20% by microscopical examination, while molecular identification revealed that 70% of the examined samples were positive for the parasite (Table.1).

**Table (1):** Prevalence of Sarcocystis infection in the examined frozen Indian buffalo meat by different examination methods.

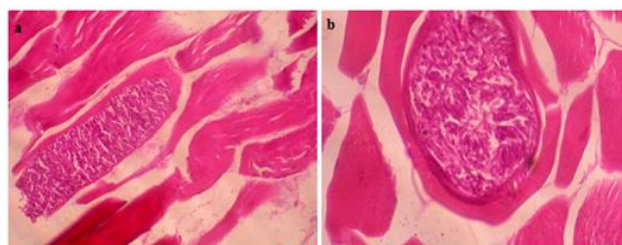
Name of Cuts	No. of examined samples	Macroscopic examination	Microscopical examination	Molecular identification (PCR)
Shoulder	16	4	3	13
Chuck	14	4	2	7
Silver Side	16	3	3	12
Knuckle	9	2	3	7
<b>Total</b>	<b>55</b>	<b>13 (23.6%)</b>	<b>11 (20 %)</b>	<b>39 (70%)</b>

**Table (2):** Sarcocystis infection in different cuts of Frozen Indian buffalo meat, by naked eye and histopathology examination only.

Name of cuts	No. of examined samples	Infection with Macroscopic cyst only		Infection with Microscopic cyst only		Mixed infection		Negative samples	
		No.	%	No.	%	No.	%	No.	%
Shoulder	16	2	12.5	1	6.25	2	12.5	11	68.75
Chuck	14	2	12.5	-	-	2	12.5	10	71.42
Silver Side	16	3	18.8	3	18.8	0	0	10	62.50
Knuckle	9	-	-	1	6.25	2	12.5	6	66.66



**Figure (1):** Macroscopic Sarcocystis in shoulder (a) and silver side (b).

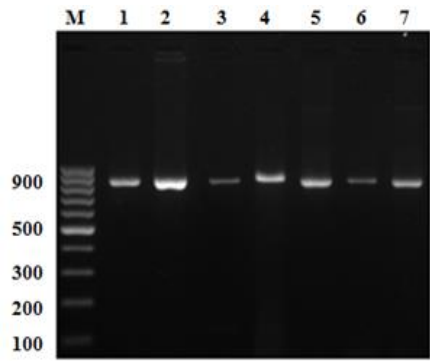


**Figure (2):** Cross section of microscopic cyst in different cuts (H & E) stain; silver side x40 (a) and shoulder x40 (b).

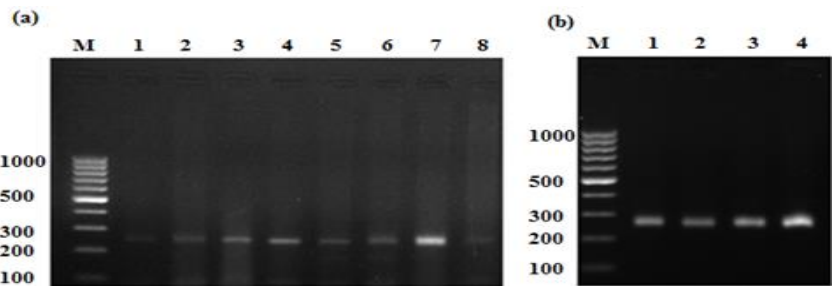
The results of using traditional method of examination showed variable prevalence of Sarcocystis infection in different cuts of Frozen Indian buffalo meat. The highest rate of cyst infections was in silver side (18.8%), and the macroscopic cyst infection in shoulder and chuck were showed the same ratio (12.5%), no infection in Knuckle was observed (Table 2). While, in case of infection with microscopic cyst, shoulder and knuckle were have the same ratio 6.25% (Table 2) and no microscopic cyst was detected in chuck.

Macroscopically, macrocysts that was isolated from frozen Indian buffalo's meat were long, wide, and white to creamy in color and could be easily separated from host tissue. They were typically

fusiform in shape (wide in middle and tapered in ends) and measured 0.2-0.5x0.3-0.6cm as shown in (Figure 1a, b). While, microcysts in histological sections stained with haematoxylin and eosin (H and E), were had blue color in muscle tissue which stained with red color. Cross section from silver side showed thin wall spindle shaped cyst parallel to muscle fibers. The cyst filled with bradyzoities without separating septa (Figure 2 a). On the other hand, Cross section from shoulder (Figure 2b) was indicated to presence thick wall, oval shaped cyst filled with bradyzoites without separating septa. Theses microcysts could be demarcated and not damage by freezing.



**Figure (3):** PCR product of (18Sr RNA gene) for *Sarcocystis Species* in DNA extracted from Indian frozen meat. Lane M: 100-bp DNA ladder and lanes 1-8: represent the positive diagnostic bands (approx.900 bp).



**Figure (4):** PCR product (approx.270 bp) of 18S rRNA gene for *Sarcocystis Species* in DNA extracted from tissue sample (a) and Macrocyt (b). Lane M: 100-bp DNA ladder.

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HM447192.15.cf.cruzi
KM434885.15.cf.cruzi
HM447193.15.cf.cruzi
TAAACCGAACGGATCGCATTATGGTCATTTTATTTGGCTGGCGATAGATCATTCCCGTTCC
TAACCCCAACGGATTTTCATTATGGTCATTTTATATGGCTGGCGATACATCATTCAAAGTTTC
TAACCGAACGGATCGCATTATGGTCATTTTATATGGCTGGCGATAGATCATTCAAAGTTTC
TAACCGAACGGATCGCATTATGGTCATTTTATATGGCTGGCGATAGATCATTCAAAGTTTC
TAACCGAACGGATCGCATTATGGTCATTTTATATGGCTGGCGATAGATCATTCAAAGTTTC
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HM447192.15.cf.cruzi
KM434885.15.cf.cruzi
HM447193.15.cf.cruzi
TGACCCCCCCCCCTTTCAACGGCCCTGTATTGGAATACCGTGGCAGTGACGGGTAAACCCGG
TGACCTATCAGCTTTCGACGTGATTTGATTGGAATACCGTGGCAGTGACGGGTAAACCCGG
TGACCTATCAGCTTTCGACGGTAGTGTATTGGAATACCGTGGCAGTGACGGGTAAACCCGG
TGACCTATCAGCTTTCGACGGTAGTGTATTGGAATACCGTGGCAGTGACGGGTAAACCCGG
TGACCTATCAGCTTTCGACGGTAGTGTATTGGAATACCGTGGCAGTGACGGGTAAACCCGG
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HM447192.15.cf.cruzi
KM434885.15.cf.cruzi
HM447193.15.cf.cruzi
AATTAGGGTTCGATTCCTTTAGGGAGCCTGAAAAACGGCTACCCACATCTAAGGAAGGCA
AATTAGGGTTCGATTCCTGGAGAGGGAGCCTGAGAAACGGCTACCCACATCTAAGGAAGGCA
AATTAGGGTTCGATTCCTGGAGAGGGAGCCTGAGAAACGGCTACCCACATCTAAGGAAGGCA
AATTAGGGTTCGATTCCTGGAGAGGGAGCCTGAGAAACGGCTACCCACATCTAAGGAAGGCA
AATTAGGGTTCGATTCCTGGAGAGGGAGCCTGAGAAACGGCTACCCACATCTAAGGAAGGCA
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HM447192.15.cf.cruzi
KM434885.15.cf.cruzi
HM447193.15.cf.cruzi
GCACGCGCGCAAATTACCAATCCTGACTCAGGGAGGTAGTGACAAGAAATAACAACACT
GCAGGCGCGCAAATTACCAATCCTGACTCAGGGAGGTAGTGACAAGAAATAACAACACT
GCAGGCGCGCAAATTACCAATCCTGACTCAGGGAGGTAGTGACAAGAAATAACAACACT
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GCAGGCGCGCAAATTACCAATCCTGACTCAGGGAGGTAGTGACAAGAAATAACAACACT
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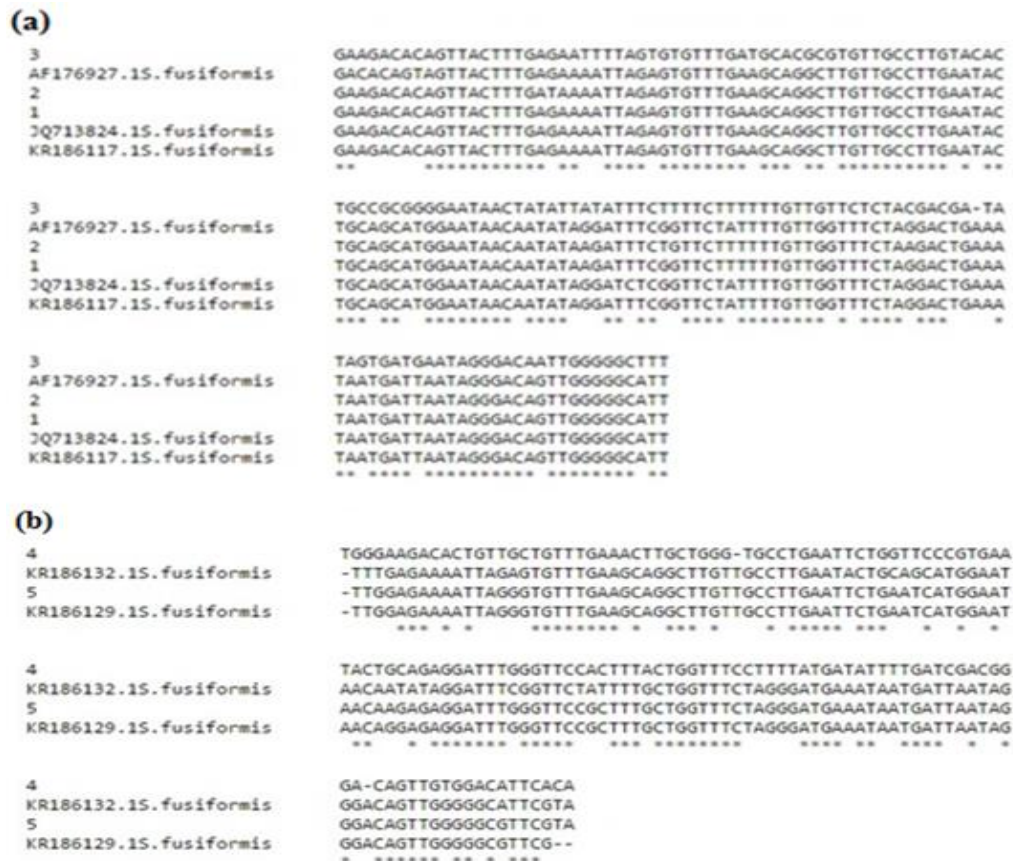
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HM447192.15.cf.cruzi
KM434885.15.cf.cruzi
HM447193.15.cf.cruzi
GGAAATTTTATTTCCAGTGACTGGAATGATGGGAATTTAAACCCCTTTTCAGAGTAAACAAT
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GGAAATTTTATTTCTAGTGATTGGAATGATGGGAATTTAAACCCCTTTTCAGAGTAAACAAT
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GGAAATTTTATTTCTAGTGATTGGAATGATGGGAATTTAAACCCCTTTTCAGAGTAAACAAT
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HM447192.15.cf.cruzi
KM434885.15.cf.cruzi
HM447193.15.cf.cruzi
TGGAGGGCAAGTCTGGTGCCAGCAGCCCGGTAATTCAGCTCCAATAGCGTATATTTAAA
TGGAGGGCAAGTCTGGTGCCAGCAGCCCGGTAATTCAGCTCCAATAGCGTATATTTAAA
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TGGAGGGCAAGTCTGGTGCCAGCAGCCCGGTAATTCAGCTCCAATAGCGTATATTTAAA
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HM447192.15.cf.cruzi
KM434885.15.cf.cruzi
HM447193.15.cf.cruzi
GTTGTTGCAGTTAAAAAGCTCGCAGTTGGATGTCTGCTGGAAGCA
GTTGTTGCAGTTAAAAAGCTCGCAGTTGGATGTCTGCTGGAAGCA
GTTGTTGCAGTTAAAAAGCTCGCAGTTGGATGTCTGCTGGAAGCA
GTTGTTGCAGTTAAAAAGCTCGCAGTTGGATGTCTGCTGGAAGCA
GTTGTTGCAGTTAAAAAGCTCGCAGTTGGATGTCTGCTGGAAGCA
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**Figure (5):** DNA sequence alignment of 18S ribosomal RNA gene in tissue samples compared with the published sequences of *Sarcocystis cruzi* on genebank (accession No. HM447193.1, HM447192. and KM434885.1.), ordered according to ClustalW2Program.



**Figure (6):** DNA sequence alignment of *Sarcocystis fusiformis* 18SrRNA gene in 3 cysts samples (a) and 2 tissue samples (b), ordered according to ClustalW2Program.

First primer was amplified partial sequences of 18S rRNA as suitable marker for distinguished sarcocystis spp. The PCR revealed a product length (approximate 900bp.) as positive results for *Sarcocystis species* (macroscopic and/or microscopic) (Figure 3).

Using second primer which was specific for 18S rRNA sequence of *S. fusiformis*, was used to amplify the DNA extracted from tissue sample (Figure 4a) and macroscopic cyst, (Figure 4b), showed DNA band at approximate length of (270 bp) as positive results for *Sarcocystis species* (macroscopic) only.

The PCR purified products of both primers from isolated macroscopic cyst and frozen tissue samples were successfully sequenced in one direction using forward primer to identify sarcocystis species. The comparison of obtained sequence of the first primer from muscle frozen tissue with published data on Gene bank using NCBI Blast program, revealed that the partial sequence of 18S rRNA gene with crossponding sequence of *S.cruzi* 93% with accession No.HM447193.1, HM447192.1 and 92% with accession No.KM434885.1. The alignment of these

sequences ordered with obtained sequences according to Clustalw program showed in (Figure 5).

The sequence analysis revealed that the partial sequences of the 18S rRNA gene of *Sarcocystis* cysts isolated from frozen Indian buffalo meat by using 2<sup>nd</sup> primer were identical with the published data of *S. fusiformis* (JQ713824.1, KR186117.1: 93%, and AF176927.1: 97%) . While, the partial 18S rRNA gene sequence of frozen Indian buffalo tissue with the same primer showed similarity percentage 96% with published data of *S. fusiformis* under accession No. KR186129.1 and 91% with accession No. KR186132.1. the alignments of the obtained sequences with homology sequence on gene bank ordered according to Clustalw program showed in (Figure 6a, b).

#### 4. DISCUSSION

In this study, macroscopical and microscopical examination and molecular characterization were used to investigate *Sarcocystis* infection in frozen Indian buffalo meat. The result revealed that the percentage of infection was 23.6% using macroscopical examination and 20 % by using

microscopical examination. While, by molecular identification using 18S rRNA gene was 70%. Traditional methods for identification and characterization of Sarcocystis species based on isolated cyst morphology using gross examination and light or transmission electron microscopy (Nourollahi-Fard et al. 2009; Dubey et al. 2015). But, molecular methods have been found useful and sensitive in detecting Sarcocystis spp. (Jehle et al. 2009; Stojceki et al. 2012; Hamidinejat et al. 2015). Additionally, molecular techniques can be utilized to distinguish the morphologically comparable sarcocystis cyst in intermediate hosts if it the same or belong to different species (Dahlgren and Gjerde 2007).

Chhabra and Somantaray (2012) reported that Sarcocystis spp. is the most common protozoa infection in buffalo (*Bubalus bubalis*) in India and in general the prevalence rate is higher than in cattle (Mohanty et al. 1995; Daptardarkar et al. 2016). Also, prevalence rate of Sarcocystis species infection (macroscopic, microscopic and both) among buffaloes slaughtered in Punjab, India was 95.5% and they suggest that this higher rate was due to the owner who raised pets animal which transmit infection to buffalo, and they leave buffaloes to graze in the open and ingest sporocysts with feed and water (Dar et al. 2016). In the current study, mixed infection (macroscopic and microscopic Sarcocystis) was investigated (Table 2) in the same cuts. Also, there is variation in infection percentage of different cuts of frozen buffalo meat, which may refer to the activity of muscle. Moreover, other study showed that among 112 infected cattle, 67 cattle have multiple Sarcocystis infection (by more than one species) (Hajimohammadi et al. 2014). The macroscopic cyst spindle shape, creamy color and easily isolated from muscle mass and not damaged by freezing. This was nearly similar to previous description of *S. fusiformis* (El-Dakhly et al. 2011; Metwally et al. 2014; Dubey et al. 2015). On the other hand, histopathological technique revealed microscopic cyst in different examine cuts with thin and thick cyst wall as described by Daptardarkar et al. (2016).

Molecular diagnostic techniques have been used for specific determination of Sarcocystis spp. As the variable regions of the 18S rRNA gene have been shown to be good genetic markers for species-specific detection of Sarcocystis spp. (Fischer and Odening 1998; Yang and Zuo 2000). In this study, we amplify partial sequence of 18S rRNA gene from DNA extracted from tissue samples and obtained a product of approximately 900 bp in

length by using genus-specific primer (18S9L and 18S1H) similarly as in previous studies (Li et al. 2002; Hamidinejat et al. 2015). The result of alignment of DNA sequences of this product revealed infection of the frozen buffalo meat with *S. cruzi* with accession No.HM447193.1 and HM447192.1. As, *S. cruzi* is the one of the main sarcocystis species infect cattle and known to cause considerable morbidity and mortality (Wee and Shin 2001). However, previous studies reported that water buffalo used as an intermediate host to *S. cruzi* and infection is not bounded to cattle only (Li et al. 2002; Jehle et al. 2009; Xiang et al. 2011; Metwally et al. 2014; Gjerde et al. 2016). As, water buffaloes are usually infected with *Sarcocystis fusiformis*, *Sarcocystis dubeyi*, *Sarcocystis levinei*, and *Sarcocystis buffalonis* (Dubey et al. 1989; Jehle et al. 2009; Abu-Elwafa et al. 2015). In this study, the sequence analyzing of 18S rRNA gene obtained from isolated macroscopic cysts and tissue sample was performed and compared with the reference sequences of *Sarcocystis* species in the GenBank which identified that these sequences were homology with the published data of *S. fusiformis*. The *Sarcocystis fusiformis* is considered nonpathogenic but they produce macrocysts which causes many economic losses due to its effect on meat quality (Hamidinejat et al., 2015).

To our knowledge there is no data to determine the species of microscopic and macroscopic sarcocysts in frozen buffaloes meat. So, it is the first time that multiple Sarcocystis infections could be identified in frozen buffalo meat which imported from other country for eating by consumer. In conclusion, the methods described in the current study would be useful for detection of sarcocystis infection especially, using of conventional PCR with specific primer for genus of sarcocystis and for *S. fusiformis* as it the most common in buffalo showed to be more accurate and less time consuming method to identify different Sarcocystis spp. in imported frozen meat.

## 5. REFERENCES

- Abu-Elwafa, S.A., Al-Araby, M.A., Abbas, I.E.A. 2015. Sarcocystis fusiformis (Railliet, 1897) infecting water buffaloes (*Bubalus bubalis*) in Dakahlia Province, Egypt. IJAR. 3: 116-120.
- Bancroft, J.D., Stevens, A. 1990. Theory and Practice of Histological Techniques, Churchill Livingstone, Edinburgh.
- Chhabra, M.B., Samantary, S. 2012. Sarcocystis and sarcocytosis in India: status and emerging perspectives. J. Parasit Dis. 37: 1-10.

- Claveria, F.G., Cruz, M.J., Lim, R.S. 2000. Sarcocystis spp. infection in Philippine water buffaloes (*Bubalus bubalis*). Southeast Asian J. Trop. Med. Public Health 31:44-47.
- Dahlgren, S., Gjerde, B. 2007. Genetic characterization of six Sarcocystis species from reindeer (*Rangifer tarandus tarandus*) in Norway based on the small subunit rRNA gene. Vet Parasitol. 146:204–213.
- Daptardarkar, M., Singh, B.B Aulakh, R.S. Gill. J.P.S. 2016. Prevalence and first molecular identification of Sarcocystis species in cattle and water buffaloes in India. Acta Parasitologica. doi: 10.1515/ap-2016-0069.
- Dar, K.H., Bhat, M.M. Shafi, M. 2016. Prevalence and organwise distribution of sarcocystosis in buffaloes of Mohali, Punjab. J. Parasit. Dis, 12639-016-0796-z.
- Dubey, J.P., Hilali, M. Van Wilpe, E., Verma, S.K., Calero-bernal, R. Abdel-Wahab, A. 2015. Redescription of Sarcocystis fusiformis sarcocysts from the water buffalo (*Bubalus bubalis*). Parasitology 142:385-94.
- Dubey, J.P., Speer, C.A., Fayer. R. 1989. Sarcocystosis of Animals and Man. CRC Press, Boca Raton, Florida, pp 1–215.
- El-Dakhly, K.M., El-Nesr, K.A., El-Nahass, E., Hirata, A., Sakai, H., Yanai, T. 2011. Prevalence and distribution patterns of Sarcocystis spp. in buffaloes in Beni-Suef, Egypt. Trop. Anim. Health. Prod. 43:1549–1554.
- Fischer, S., Odening, K.1998. Characterization of bovine Sarcocystis species by analysis of their 18S ribosomal DNA sequences. J. Parasitol. 84:50–54.
- Food and Agricultural Organization FAO. 2009. FAOSTAT database. Food and Agricultural Organization of United Nations. <http://faostat.fao.org/> Accessed 13.03.12.
- Gjerde, B., Hilali, M. Abbas, I.E. 2016. Molecular differentiation of *Sarcocystis buffalonis* and *Sarcocystis levinei* in water buffaloes (*Bubalus bubalis*) from *Sarcocystis hirsuta* and *Sarcocystis cruzi* in cattle (*Bos taurus*). Parasitol. Res. 115:2459–2471.
- Hajimohammadi B., G. Eslami, A. Zohourtabar, A. Dehghani, A. Oryan, T.H. Pourmirzaei, and F. Radouani. 2014 High Occurrence of Sarcocystis Cysts in Meat Produced in Yazd, Central Iran. J. Food Qualit. Haz. Cont. 1:95-101.
- Hamidinejat, H., Jalali, M.H.R. Gharibi, D. Molayan, P.H. 2015 Detection of Sarcocystis spp. in cattle (*Bos taurus*) and water buffaloes (*Bubalus bubalis*) in Iran by PCR–RFLP. J Parasit Dis. 39:658–662.
- Huong, L.T., Dubey, J.P., Nikkilä, T., Uggla, A. 1997. Sarcocystis buffalonis n.sp. (Protozoa: Sarcocystidae) from the water buffalo (*Bubalus bubalis*) in Vietnam. J Parasitol. 83:471-474.
- Huong, L.T., Uggla, A. 1999. Sarcocystis dubeyi n. sp. (Protozoa: Sarcocystidae) in the water buffalo (*Bubalus bubalis*). J. Parasitol. 85:102-104.
- Jain, P.C., Shah, H.L. 1985. Cross-transmission studies of *Sarcocystiscruzi* of the cattle to the buffalo calves. Indian J Anim. Sci. 55:27–28.
- Jehle, C., Dinkel, A., Sander, A., Morent, M., Romig, T., Luc, P.V., De, T.V., Thai, V.V. , Mackenstedt, U. 2009. Diagnosis of Sarcocystis spp. in cattle (*Bos taurus*) and water buffalo (*Bubalus bubalis*) in Northern Vietnam. Vet Parasitol. 23:314-320.
- Li, Q.Q., Yang, Z.Q., Zuo, Y.X., Attwood, S.W., Chen, X.W., Zhang, Y.P. 2002. A PCR-based RFLP analysis of Sarcocystis cruzi (Protozoa: Sarcocystidae) in Yunnan Province, PR China, reveals the water buffalo (*Bubalus bubalis*) as a natural intermediate host. J. Parasitol. 88: 1259–1261.
- Metwally, A.M., Abd Ellah, M.R., AL-Hosary, A.A., Omar, M.A. 2014. Microscopical and serological studies on Sarcocystis infection with first report of S. cruzi in buffaloes (*Bubalus bubalis*) in Assiut, Egypt. J Parasit Dis. 38:378–382.
- Mohanty, B.N., Misra, S.C., Panda, D.N., Panda, M.R. 1995. Prevalence of Sarcocystis infection in ruminants in Orissa. Ind.Vet.J. 72: 1026–30.
- Nourollahi-Fard, S.R., Asghari, M., Nouri. F. 2009. Survey of Sarcocystis infection in slaughtered cattle in Kerman, Iran. Trop. Anim. Health Product. 41: 1633-1636.
- Pritt, B., Trainer, T., Simmons-Arnold, L., Evans, M., Dunams, D., Rosenthal, B.M. 2008. Detection of sarcocystis parasites in retail beef: a regional survey combining histological and genetic detection methods. J Food Prot. 71:2144-2137.
- Sambrook, J., Russell, D.W. 2001. Molecular cloning: a laboratory manual third edition, Coldspring Harbor Laboratory press, New York.
- Stojecki, K., Karamon, J., Sroka, J., Cencek, T. 2012. Molecular diagnostics of Sarcocystis spp. infections. Polish J. Vet. Sci. 15: 589-596.
- Thompson, J.D., Higgins, D.G., Gibson, T.J., Clustal, W. 1994. Improving the sensitivity and progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucl. Acids Res. 22:4673–4680.
- Wee, S.H., Shin, S.S. 2001. Experimental induction of the two-host life cycle of Sarcocystis cruzi between dogs and Korean native calves. Korean J Parasitol. 39:227-32.
- Xiang, Z., He, Y., Zhao, H., Rosenthal, B.M., Dunams, D.B., Li, X., Zuo, G. Feng, Y., Cui, L., Yang, Z. 2011. Sarcocystis cruzi: comparative studies confirm natural infections of buffaloes. Exp. Parasitol. 127:460-6.
- Yang, Z.Q., Zuo. Y.X. 2000. The new views of the researchers on cyst forming coccidia species including Sarcocystis by using the molecular biological techniques. Chin J. Parasitol. Dis. 18:120–126.