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MOLECULAR IDENTIFICATION OF *SARCOCYSTIS FUSIFORMIS* AND *S. MOULEI* INFECTING WATER BUFFALOES (*BUBALUS BUBALIS*) IN SOUTHERN IRAQ

Huda Ghanim Dakhil[1]*, Basim Hashim Abdallah[1] and Fawzia Ali Abdallah[2]

[1]Department of Biology, Education College for Pure Science, Basrah University.

[2]Department of Microbiology, Veterinary Medicine College, Basrah University.

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***Corresponding Author'**
Dr. Huda Ghanim Dakhil
Department of Biology, Education College for Pure Science, Basrah University.

ABSTRACT

The current study was aimed to screen infection rates and genetic identification of the macroscopic *Sarcocystis* spp. infecting Iraqi water buffalos. *Sarcocystis* were grossly examined through meat inspection in slaughterhouse, followed by molecular identification using PCR, sequencing and phylogenetic analysis of the 18S rRNA gene was conducted. An over all rates of *S. fusiformis* and *S. Moulei* infection in Iraqi water buffaloes were 2.8% and 0.1% respectively. According to age, higher rates of *Sarcocystis* spp. infection was observed in older animals (>4 years) compare to that observed in younger animals (≤4 years). In case of old-aged animals 5.5% (24 /436) and 0.1% (1/904) were found to be infected with *S. fusiformis* and *S. moulei* respectively. Comparison of the obtained sequences of the local tested samples of *S. fusiformis* and *S.moulei* with those previously reported for different *Sarcocystis* spp. those were recorded in Genbank, revealed that all the tested specimens represented *S. fusiformis* and only one specimen was positive for *S.moulei*. Moreover, sequence alignments of the partial 18S rRNA gene ascertained the presence of genetic difference revealing the heterogenic nature of the gene among different strains of the same *Sarcocystis* species in relation to the geographic distribution. This is the first study for the evaluation of the PCR technique for apply genetic identification of the macroscopic *Sarcocystis fusiformis* infecting Iraqi water buffalos and the current study reported for the first time infection of water buffalo with *S.moulei* in the world.

KEYWORDS: *Sarcocystis fusiformis* in Iraq, *Sarcocystis moulei* in water buffaloes (*Bubalus bubalis*).

INTRODUCTION

Sarcocystis was reported in 1843 by Miescher in striated muscles of a house mouse, without a scientific name. similar structures were found in pig muscle in 1865, name *Sarcocystis meischeriana* was proposed to identify them. *Sarcocystis* is one of the most prevalent parasites of livestock (Dubey *et al*., 1989). *Sarcocystis* species are intracellular protozoan parasites, life cycle based on intermediate and definitive hosts . *Sarcocystis* spp. Specificity for Intermediate and definitive hosts For example, sporocysts of *S.hominis* infect cattle but not pigs in another side *S. suihominis* infect pigs but not cattle. Sporocysts of *S.ovicanis* from dogs and *S . ovifelis* from cats infect sheep but not water buffalo or goats. Sporocysts of *S. hirsute* from cats infect cattle but not goats, *S. cruzi* from dogs can infect cattle, water buffalo, and bison (Chen *et al*., 1999). Some species of *Sarcocystis* can cause reduced weight gain, poor feeding efficiency, fever, anorexia, muscle weakness, reduced milk yield, anemia, abortion, and death in intermediate hosts as cattle, goats, sheep, and swine. Certain species infect humans and can cause digestive trouble such as vomiting, nausea and diarrhea (Dubey *et al*., 1989b). The conventional method of distinguishing the different *Sarcocystis* spp. is by observing the structure of the cyst wall under light microscopy or transmission electron microscopy and combining these data with information on the lifecycle (host species involved). However, such methods are not suitable for the identify cation of those *Sarcocystis* taxa which show very little morphological differences or for studies of host range. In other cases natural differences within *Sarcocystis* spp. may have led to the unnecessary distinction of new taxa, which closely resemble known species, and their consideration as possible new species (Yang *et al*., 2001).

Molecular methods have been used for species identification were identified by sequencing 18S rRNA gene PCR products (Fayer *et al*., 1982). The variable regions of the 18S rRNA gene have been shown to be good genetic markers for characteristic certain species of *Sarcocystis* (Fischer and Odening, 1998; Yang and Zuo, 2000).

The current study aimed to screen prevalence of *Sarcocyctis* infection and apply genetic identification of the macroscopic *Sarcocystis* spp. infecting Iraqi water buffalos in southern Iraq.

MATERIALS AND METHODS

Source and collection of samples

Nine hundred and four water buffaloes, aged 0.5-7 years, slaughtered at the Misan province abattoir in southern Iraq, were used in the survey for the presence of *Sarcocystis* spp. during the period from February 2015 to January 2016 According to age, buffaloes were categorized into two groups, the first 4 years and less and the second included animals of more than 4 years. The esophagus muscles of the slaughtered animals were collected and placed in properly labelled plastic bags, then transported to the Parasitology Laboratory of the Education College for Pure Science, Basrah University. The muscle samples were kept in the refrigerator prior to the examination for *Sarcocystis* spp. The presence of the *Sarcocystis* spp. was evidenced through a naked eye examination of the muscles.

Morphologic identification

Esophageal muscles were examined by the naked eye to detect macroscopic *Sarcocystis* spp. Macroscopic visible *Sarcocystis* spp. were carefully isolated from muscle samples and their shape and size was determined.

Molecular identification

The molecular analysis was used for confirmation of species identification, specificity to intermediate host and the relation of cysts shape with species identification. The *Sarcocystis* spp. were isolated from muscle tissues of slaughtered water buffalo with sterile knife, each cyst washed 3-4 times with 0.9% NaCl as previously described by Yang *et al*. (2001). Many macroscopic *Sarcocystis* spp. were obtained from each slaughtered animal and incubated in to phosphate buffer saline (pH 7.2) and transferred to the Parasitology laboratory of the Education College for Pure Science Basrah University for further processing.

DNA extraction

DNA was extracted from *Sarcocystis* spp. obtained from each infected animal using Bioneer Blood and Tissue kit® according to manufacturer protocol.

PCR amplification

PCR reactions were applied for each DNA extract using the primer sets those were designed according to 18S rRNA sequence of *S. fusiformis*, *S. moulei* and *S. gigantea* (table 1). These primer target the 18S rRNA gene in a final reaction volume of 25 µL The PCR amplification mixture (25µl) includes tubes containing Accu Power® PCR Master mix (which contains

bacterially derived Taq DNA polymerase, dNTPs, MgCl2 and reaction buffer at optimal condensation for efficient amplification of DNA templates by PCR)2 µl of template DNA ,1 µl of each forward and reverse primers and 16 µl of nuclease free water to complete the amplification mixture to 25 µl. The PCR tubes containing amplification mixture were transferred to preheated thermocycler (Cleavere Scientific-USA) under thermocycling parameters listed in table (1). Amplified products were resolved by using 1.5% agarose gel electrophoresis containing 0.5 µl /25 ml ethidium and DNA size marker, examined using a UV trans illuminator and photographed.

Table (1): Oligonucleotide primers sequence target the 18S rRNA gene and PCR amplification condition.

***Sarcocystis* spp.**	**Primers(bp) References**	**PCR reaction condition**			
		Step	**Temprature °C**	**Time**	**cycles**
S.fusiformis	Forward – 5′CGCCCTTTTAGTGAGGGTGT3′ Revers - 5′ TACGAATGCCCCCAACTGTC3 ′ (490 pb) El-Seify *et al*. 2014	Denaturation Annealing Extension Terminal	94 94 60 72 72	5 min 30 sec 45 sec 30 sec 10 min.	35
S. moulei	Forward- 5′GCACTTGATGAATTCTGGCA 3′ Reverse- 5′CACCACCCATAGAATCAAG 3′ (637-bp) Motamedi *et al* ., 2010	Denaturation Denaturation Annealing Extension Terminal	94 94 60 72 72	5 min 2 min 30sec 5 min 2 min	35
S. gigantea	Forward - 5′TTCTATGGCTAATACATGCG 3′ Reverse - ′CCCTAATCCTTCGAAACAGGA 3′ 5 (964 bp) Farhang –pajuh *et al* ., 2014	Denaturation Denaturation Annealing Extension Terminal	94 94 57 72 72	5 min. 60 sec 40 sec 60 sec 5 min	35

DNA sequencing, sequence alignment and phylogenic analysis

PCR amplicons were sent to Macrogen company labrotary in Korea. The sequence of the clones was determined from forward direction (5′-3′). The 18S rRNA gene fragments were sequenced using the same primers used in PCR. 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 *Sarcocystis* isolates, then between the sequence alignments of the local species with those of previously published species of *S. fusiformis* and *S. moulei* to determine the percentages of variability and similarities among them. To reconstruct the phylogenetic relationship 18S rRNA PCR

products representing each isolate were included in a comparative analysis with those formerly published in the NCBI using BLAST (http://blast.ncbi.nlm.nih.gov/), on the basis of highly similar sequences available on Genbank data base. The evolutionary history was investigated by using the UPGMA method (Saitou & Nei 1987) the optimum tree with the total of branch length = 2.91769427 was shown in Fig 10. The tree is drawn to scale, with branch lengths with in the same units as those of the evolutionary distances confirmed the phylogenetic tree. It's distances were computed utilization the Maximum Composite Likelihood method (Tamura Nei & Kumar 2004)and are in the units of the number of base substitutions per site.

Statisticallanalysis

To demonstrate any associationl between results, the Pearson'schi-squared test withlYates correction were used with the limit of significance being setlat 5%. Statistical analysis is done by using SPSS software lversion 11.

RESULTS

Morphologic Identification

Sarcocystis spp. cysts were macroscopic, opaque white in color, measured 2mm - 2.5 cm X 1mm - 0.5 cm and located sometimes under the serosal sheet or deeply situated within the esophageal musculature. Two shapes of cyst were observed in the present study, the cylindrical and the oval cysts (Fig.1,2,3,4).

Prevalence of *Sarcocystis* spp. infection

The results of current study revealed that an over all rates of *S. fusiformis* and *S. moulei* infiction in Iraqi water buffaloes were 2.8% and 0.1% respectively. According to age, higher ratio of *Sarcocystis* spp. infection was observed in older animals (>4 years) compare to that observed in younger animals (≤4 years). In case of old-aged animals, 5.5% (24 /436) and 0.1% (1/904) were found to be infected with *S. fusiformis* and *S. moulei*, respectively. *S. fusiformis* infection ratio in younger animals was 0.2% (1/468) while *S. moulei* was not detected in those animals. *S. gigantea* was not detected in all morphological and molecular tested cyst samples of buffaloes at both age groups. The Statistical analysis results revealed that there was significant difference between the two age groups of animals concerning the number of *S. fusiformis* infected buffaloes (X^2: 21.572 df :1 P: 0.00000341) but this difference was not considered to be Statistically significant in case of *S. moulei* (X^2:1.075 df :1 P: 0.29981879). In general the difference in the distribution of studied *Sarcocystis* spp.

among buffaloes at both age groups was considered to be extremely statistically (X^2:113.35 df :5 P: 0) significant. Mixed infections with both species of *Sarcocystis* was found in 0.2% (1/436) of older animals (>4 years) (Table 2).

Fig 1: Macroscopic *Sarcocystis* spp. Cysts.

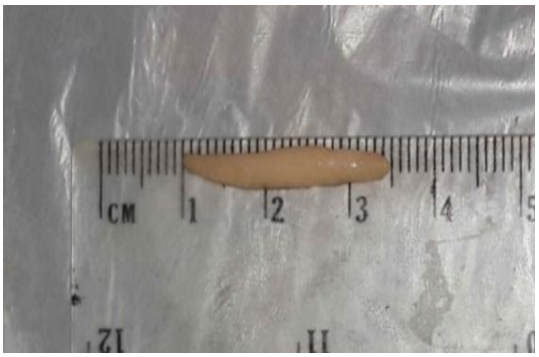

Fig 2: Macroscopic *Sarcocystis* spp. cylindrical Cysts

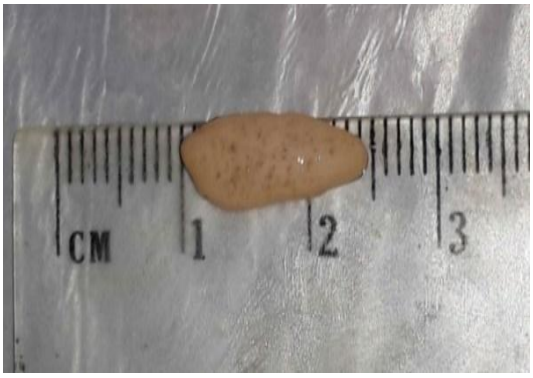

Fig .3 Macroscopic *Sarcocystis* spp. oval Cysts

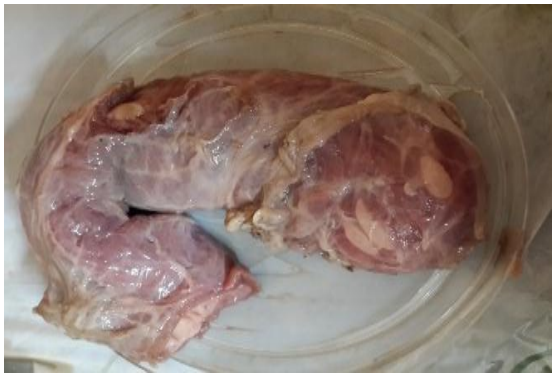

Fig .4. Macroscopic *Sarcocystis* spp.in esophageal muscle of water buffalo.

Table 2: Prevalence of *Sarcocystis* spp. in Iraq water buffaloes (*Bubalus bubalis*) relative to age during the period extending from February 2015 to January 2016.

Age groups	Examined animals n.(%)	Animals infected with			
		S. fusiformis * n.(%)	*S.moulei* ** n.(%)	*S. gigantea* *** n.(%)	Mixed infection n.(%)
4 years and lest	468 (51.8)	1 (0.2)	0 (0)	0 (0)	0 (0)
More of 4 years	436 (48.2)	24 (5.5)	1 (0.2)	0 (0)	1 (0.2)
Total n.(%)	904 (100)	25 (2.8)	1 (0.1)	0 (0)	1 (0.1)

* X^2:21.572 df :1

P: .00000341

** X^2:1.075

df:1 P:0.29981879

*** X^2:113.35 df :5

P: 0

Molecular Identification

PCR based species identification

Despite the different shapes of macrocystes but current study proved that there was no relation between cyst shape and species identification. The genetic analysis of DNA extract which was obtained from cylindrical and oval *Sarcocystis* revealed same *Sarcocystis* spp. (*S. fusiformis* or *S.moulei*). The result of PCR amplification was confirmed by electrophoresis as the strands of the DNA which are resulted from successful binding between specific primer of *S. fusiformis* and *S.moulei* and the extracted DNA, appeared as single band on 2.5% agarose and under UV. Illuminator using Ethidium bromide as specific DNA stain. Only bands with expected size (390 and 600bp respectively) were observed while no band was observed in cases of *S. gigantean*. (fig. 5 and 6).

Sequencing analysis and phylogenetic tree

The results of BLAST comparative analysis, revealed that the partial 18S rRNA gene sequences of two local tested isolates of *S. fusiformis* and *S. moulei* revealed that there were two strains of *S.fusiformis* is including *S. fusiformis* strain 3f5 and *S.fusiformis* (fig.7 and 8). The sequence identity between the current isolate of *S. fusiformis*is strain 3f5 18S and *S. fusiformis* is with published data of *S. fusiformis*is (accession number AF176927 and JQ713824 respectively) were (93%, 91% respectively). Same sequence identity sequence identity (99%) was observed between *S. moulei* isolate from Misan water buffalo and Iran goats and sheep (accession number KF489430). The sequence identity between the current isolate of *S. fusiformis* is and published data of *S. fusiformis* is showed variables rates of simillarity to that reported in china, india, Egypt, Iran and Sweden (Table 3).

Only one strains of *S.moulei* was confirmed by gene sequence analysis (fig.9) which showed 99% simirality with *S. moulei* strain Ecy1 recorded in goats of Iran, Australia and Sweden (Table 3). The analysis involved fifteen nucleotide sequences. All positions containing gaps and missing information were eliminated. There have been a complet 168 positions with in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura *et al*., 2013).

The phylogenetic analysis of the 18S rRNA sequences showed that the two Iraqi strains of *S. fusiformis* placed in a group with the previously reported *S.fusiformis* in India (JQ713824), China (AF176926), AF176927) and Egypt (KR186131, KR186121 and KR186127). Furthermore, this group was associated with other group of *S. fusiformis* previously reported in Iran (HQ703791) and Sweden (U03071). *S. moulei* local isolates was more associated with group of *Sarcocystis moulei* isolated from Iran goats (accession number KF489430, KF489423) (Motamedi *et al.*,2010), together with *S. moulei* isolated from Australia sheep with accession number L76473 (Fig. 10).

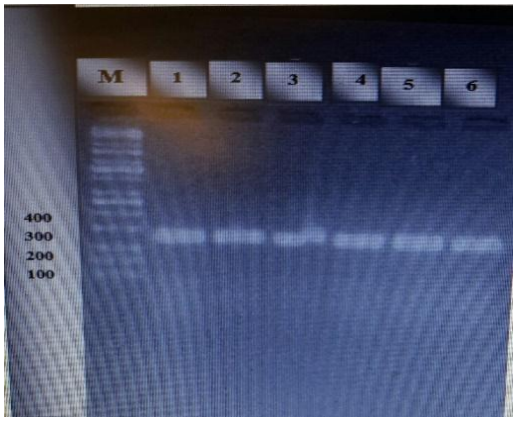

Fig. 5: Electrophoresis of the 18S rRNA gene used for PCR assay for the local tested isolates of *S. fusiformis*. Lane M100-bp ladder = DNA marker. Lanes 1-5 represent the positive diagnostic bands of the tested specimens.

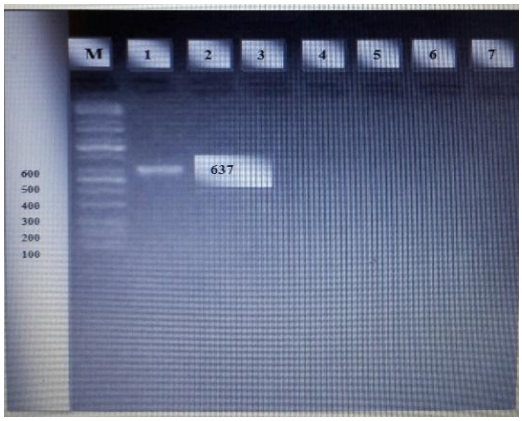

Fig. 6: Electrophoresis of the 18S rRNA gene used for PCR assay for the local tested isolates of *S . moulei* Lane M100-bp ladder = DNA marker. Lanes 1-5 represent the positive diagnostic bands of the tested specimens.

Score	Expect	Identities	Gaps	Strand
294 bits (159)	4e-76	184/196 (94%)	2/196 (1%)	Plus/Plus

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Query  1    TATTATTATATTAGGTTGATTCCTAATATGGTAATAATTATTTTTGGGAAGACACAGTAG 60
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  698  TATTATTATATTAGGTTGATTCCTAATATGGTAATAATTATTTTTGGGAAGACACAGTAG 757

Query  61   TTACTTTAAGAAAATTGTATTGTTTGAACCAGGCTTGTTGCCTTGAGTACTGCAGCATGG 120
           |||||||  ||||||||   | ||||||||  ||||||||||||||||  ||||||||||||
Sbjct  758  TTACTTTGAGAAAATTAGAGTGTTTGAAGCAGGCTTGTTGCCTTGAATACTGCAGCATGG 817

Query  121  AATAACAATATATGATTTCTGTTCTATTTTGTTGTTTTCTAGGACTGAAATAATGATTAG 180
           ||||||||||||  ||||||  ||||||||||||||  ||||||||||||||||||||||||
Sbjct  818  AATAACAATATAGGATTTCGGTTCTATTTTGTTGGTTTCTAGGACTGAAATAATGATTA-  876

Query  181  ACA-GGACAGTTGGGG  195
           |  |  ||||||||||||
Sbjct  877  ATAGGGACAGTTGGGG  892
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Fig 7: Sequence alignment of 18S rRNA gene for local tested isolates *S. fusiformis* strain 3f5 partial sequence .Sequence ID: gb|AF176927.

Score	Expect	Identities	Gaps	Strand
283 bits (153)	8e-73	171/179 (96%)	3/179 (1%)	Plus/Plus

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Query  61   CTTTGAGAAAATTATTGAGTTGTTTGAAGGCGGCTTGTTGCCTTGAATACTGCAGGATGG  120
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Sbjct  659  CTTTGAGAAAATTA--GAGT-GTTTGAAGCAGGCTTGTTGCCTTGAATACTGCAGCATGG  715

Query  121  AATAACAATATAGGATTTCTGTTCTATTTTGTTGGTTTCTAGGACTGAAATAATGATTA  179
           ||||||||||||||||  ||  ||||||||||||||||||||||||||||||||||||
Sbjct  716  AATAACAATATAGGATCTCGGTTCTATTTTGTTGGTTTCTAGGACTGAAATAATGATTA  774
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Fig. 8: Sequence alignment of 18S rRNA gene for local tested isolates *S. fusiformis*. partial sequence. Sequence ID: gb|JQ713824.1|

Score	Expect	Identities	Gaps	Strand
472 bits (255)	2e-129	264/268(99%)	2/268 (0%)	Plus/Plus

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Query  121  AGGGGCTCGAAGACGATCAGATACCGTCGTAGTCTTAACCATAAACTATGCCGACTAGAG  180
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Query  18   ATAGGAAAATGTCGC--TGTTGACTTCTCCTGCACCTTATGAGAAATCAAAGTCTTTGGG  238
           |||||||||||||  |  ||||||||||||||||||||||||||||||||||||||||||
Sbjct 330   ATAGGAAAATGTCACATTGTTGACTTCTCCTGCACCTTATGAGAAATCAAAGTCTTTGGG  389

Query  23   TTCTGGGGGGAGTATGGTCGCAAGGCTG  266
           ||||||||||||||||||||||||||||
Sbjct 390   TTCTGGGGGGAGTATGGTCGCAAGGCTG  417
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Fig 9: Sequence alignment of 18S rRNA gene for local tested isolates *S. moulei* strain Ecy1, partial sequence.Sequence ID: gb|KF489430.1|

DISCUSSION

Sarcocystosis is common within a wide hosts of vertebrates as well as humans (Abdel-Ghaffar and Al-Johany, 2002). For the first time, the macroscopic *S. fusiformis* and *s.moulei* infection was detected in Iraqi water buffaloes with prevalence rate of 2.8 and 0.1% respectively. One previous study in North of Iraq, (Al-Nakshabandi, 2008), has been reported total percentage (96%) of *Sarcocystis* spp. infection in Bovine muscles samples depending on morphological characteristics of *Sarcocystis* spp. without detailed identification which revealed *Sarcocystis* spp. In previous studies throughout the world, Khalifa *et al*. (2008)

examined specimens from tongue, heart, esophagus and skeletal muscles of 100 water slaughtered buffaloes in Egypt, revealing an infection rate of 28% with *S. fusiformis* only. El-Dakhly *et al*. (2011) examined 379 water buffaloes, in Egypt and found that 299 were infected with overall prevalence of 8.9%. Beside that El-Morsy (2010) and Salah *et al*. (2015) reported *S. fusiformis* infection ratio (13.2 and 8.72% respectively) in Egyptian water buffaloes. Oryan *et al.* (2011) in Iran reported that 3% of water buffaloes infected with *S. fusiformis*.

Based on age, the examined animals were categorized into two groups in the current study. Higher Infection rates with both *S. fusiformis* and *S. moulei* were 5.5 and 0.2% respectively in animals at age group >4years, This may be due to that the *Sarcocystis* spp. need longer time to appear macroscopically. Inagreement with the present resuls El-Dakhly *et al*. (2011) found Infection rates with both macroscopic of *S. fusiformis* and microscopic of *S. levinei* were 74.5%, 82.3% and 81.2% in animals aged 1.5-2 years, adult females aged 2-5 years and females aged over 5 years, respectively. El-Seify *et al*. (2014) reported higher *S. fusiformis* infection ratio (68.2%) in old -aged Egyptian water buffaloes compare to yonger water buffalo infection ratio (17.2%).

Despite *S.moulei* infection specificity for goats and sheep as naturel intermediate hosts, the current study reported for the first time infection of water buffalo with *S.moulei* in the world. Furthermore *S.moulei* was not reported as a causative agent of goats or sheep sarcocystosis in Iraq or world , only one recent Iraqi study indicated that *S. tenella* detected in 60(100%) samples of sheep muscle and esophagus depending on PCR analysis (Ayoub *et al.*,2016).

The different regions of the 18S rRNA gene have been shown to be good genetic markers for distinguishing certain species of *Sarcocystis* (Yang and Zuo, 2000; Li *et al*., 2002; Dahlgren and Gjerde, 2007; Jehle *et al*., 2009). In addition Holmdahl *et al*. (1999) mentioned that 18S rRNA genes have been extensively used as suitable targets for the accurate identification of the closely related of *Sarcocystis* species as well as phylogenetic analyses. Accordingly the present study targeting partial sequencing of 18S rRNA gene to identify *S. fusiformis* and *S. buffalonis* in water buffaloes in Iraq with the first molecular characterization of them in the country. But the current results of molecular analysis revealed positive result for *S. fusiformis* and *S. buffalonis* negative result in water buffalo. In contrast, both species were reported from water buffaloes worldwide including China (Li *et al*., 2002; Yang *et al*., 2001). Vietnam (Jehle *et al*., 2009) and Iran (Oryan *et al.*, 2011).

According to achieved sequences of 18S rRNA gene and the phylogenic tree analysis The two strains of identified *S. fusiformis* strain 3f5 and *S. fusiformis* were genetically and closely related to *S. fusiformis* (accession number AF176927, isolated from water buffalo in China) and *S. fusiformis* (accession number gb|JQ7138242, isolated from water buffalo in India) .beside that high sequence identity was observed between *S. moulei* isolated from local water buffalo and *S. moulei* isolate of sheep in Iran (accession number KF489430).The sequence identity among the current isolates (*S. fusiformis* and *S. moulei*) and published data of these two *Sarcocystis* showed variables rates of simillarity to that reported in china, india, Egypt, Iran and Sweden. Furthermore the phylogenetic tree showed two distinct groups of *Sarcocystis* spp. among the water buffalo in Iraq. the two Iraqi str*ains* of *S. fusiformis* placed in a group with these previously reported *S.fusiformis* and *S. moulei* local isolates was more associated with group of *S. moulei* isolated from sheep and goats in Iran and Australia However, all these *Sarcocystis* spp. are related to each other as they utilize water buffaloes as intermediate host. Moreover, the genotypic similarity of these species was also reflected in their close phenotypic resemblance (both species are macroscopic). The 18S rRNA gene differentiate by the presences of various loci of polymorphism between the of *Sarcocystis* spp. and within same species (Holmdahl *et al*., 1999; Oryan *et al*., 2011). Therefore, examination of more *Sarcocystis* from other geographic locations and sequencing at more genetic loci (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* spp. is that water buffaloes in different geographic regions may harbor various species 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 reproduce of this gene that were amplified from various merozoites within the *Sarcocystis* spp.

In conclusion Higher infection rates with both macroscopic *S.fusiformis* and *S. moulei*. within older age water buffaloes were observed. and the present study included the first molecular identification and first record for the infection of Iraqi water buffaloes with *S. fusiformis* and *S. moulei*.

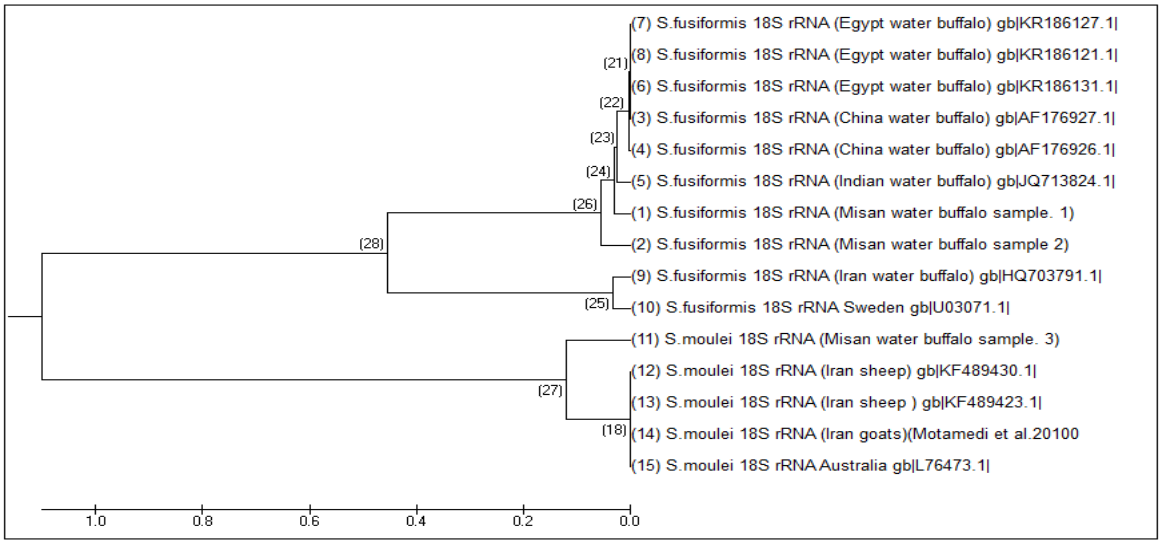

Fig 10: The phylogenetic tree of *S. fusiformis* and *S. moulei* 18S rRNA partial gene sequence in compared with highly related identity to gene bank strains by using Mega 6.

Table 3: Sequencing analysis of *S. fusiformis* and *S. moulei* 18S rRNA gene as compared with highly related identity to gene bank strains by using BLAST software in NCBI.

ACCESSION	Description	Compatibility	E-Value	Score	Range
gb|AF176927.1| AF176927	*Sarcocystis fusiformis* strain 3f5 18S ribosomal RNA gene, partial sequence/China (Misan water buffaloes)	93%	9e-76	293	698-892
gb|KR186131.1|	*Sarcocystis fusiformis* isolate Bb4.5 clone aberrant 4.5.6 18S ribosomal RNA gene, partial sequence/Egypt	93%	1e-74	289	737-931
gb|KR186127.1|	*Sarcocystis fusiformis* isolate Bb4.5 clone aberrant 4.5.1 18S ribosomal RNA gene, partial sequence/Egypt	93%	1e-74	289	737-931
gb|KR186121.1|	*Sarcocystis fusiformis* isolate Bb5.1 clone 3 18S ribosomal RNA gene, complete sequence/Egypt	93%	1e-74	289	745- 939
gb|AF176926.1|AF176926	*Sarcocystis fusiformis* strain 2f1 18S ribosomal RNA gene, partial sequence/China	93%	1e-74	289	698-892

gb|JQ713824.1|	*Sarcocystis fusiformis* 18S ribosomal RNA gene, partial sequence/India (Misan water buffaloes)	91%	8e-70	273	599-790
gb|HQ703791.1|	*Sarcocystis fusiformis* strain Ahvaz1 18S ribosomal RNA gene, partial sequence/Iran	90%	4e-68	268	563-752
gb|U03071.1|SFU03071	Sarcocystis fusiformis 18S rRNA /Sweden	90%	2e-65	259	745-940
gb|KF489430.1|	*Sarcocystis moulei* strain Ecy1 18S ribosomal RNA gene, partial sequence/Iran (Misan water buffaloes)	99 %	2e-129	472	150- 417
gb|KF489432.1|	*Sarcocystis moulei* strain Ecy2 18S ribosomal RNA gene, partial sequence/Iran	99%	1e-126	462	149- 416
gb|L76473.1|	*Sarcocystis moulei* 18S ribosomal RNA gene, partial sequence / Australia	99%	1e-126	462	934-1201
gb|KF489423.1|	*Sarcocystis moulei* strain Hea2 18S ribosomal RNA gene, partial sequence/Iran	99%	1e-126	462	128 - 395

REFERENCES

1. Abdel-Ghaffar F, Al-Johany A. (A light and electron microscope study of *Sarcocystis mitrani* (sp. Nov.) infecting the skink *Scincus mitranus* in the central region of Saudi Arabia), 2002, Parasitology research, 88(2): 102-106.
2. Al-Nakshabandi A A. (Rative study on cattle sarcocystosis diagnostic techniques in dohuk governorate), 2008, Bas.J.Vet, Res., 7(2): 16-20.
3. Ayoub I A, Anas H, Faisal A. (Molecular identification of Microscopic Cysts of *Sarcocystis* in Sheep in Baghdad Province), 2016, International Journal of Advanced Research in Biological Sciences., 3(11): 200-205.
4. Chen X, Zuo Y, Zuo W. (Observation on the clinical symptoms and sporocysts excretion in human volunteers experimentally infected with *Sarcocystis hominis*), 1999, Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi (in Chinese)., 17(1): 25-7.
5. Dahlgren SS, Gjerde B. (Genetic characterization of six *Sarcocystis* species from reindeer (*Rangifer tarandus tarandus*) in Norway based on the small subunit rRNA gene), 2007; Veterinary Parasitology., 146(3-4): 204-213.

6. Dubey J P, Speer C A, Fayer R. (*Sarcocystis* of animals and man), 1989, CRC Press, Inc. Boca. Raton, Florida., 215.
7. El-Dakhly K H M, El-Nesr K H A, El-Nahass E, Hiroki S. (Prevalence and distribution patterns of *Sarcocystis* spp. In buffaloes in Beni-Suef, Egypt), 2011, Tropical Animal Health and Production, 43(8): 1549-1554.
8. El-Morsy A M. (Studies on *Sarcocystis* species infecting water buffaloes in Egypt), 2010, Unpublished M. V. Sc. Thesis, Fac. Vet. Med., Kafrelsheikh Univ.
9. El-Seify M, El-Morsy A, Hilali M, Zayed A, El-Dakhly Kh, Haridy M, Sakai H, Yanai T. (Molecular characterization of *Sacocystis fusiformis* and *Sarcocystis buffalonis* infecting Water (*buffaloes bubalus*) from Egypt), 2014, American Journal of Animal and Veterinary Sciences., 9(2): 95-104.
10. Farhang –pajuh F, Yakhchali M, Mardani K. (Molecular determination of abundance of infection with *Sarcocystis* species in slaughtered sheep of Urmia, Iran), 2014, Journal Homepage Veterinary Research Forum., 5: 181-186.
11. Fayer R, Dubey J P, Leek R G.(Infectivity of *Sarcocystis* spp. from bison, elk, moose and cattle via sporocysts from coyotes), 1982, The Journal of Parasitology., 68(4): 681-685.
12. Holmdahl O J, Morrison D A, Ellis J T, Huong L T. (Evolution of ruminant *Sarcocystis* (Sporozoa) parasites based on small subunit rDNA sequences), 1999, Mol. Phylogenet. Evol., 11(1): 27-37.
13. Khalifa R M, El-Nadi N A, Sayed F G, Omran E K. (Comparative morphological studies on three *Sarcocystis* species in Sohag, Egypt), 2008, Journal of the Egyptian Society of Parasitology., 38(2): 599-608.
14. Jehle C, Dinkel A, Sander A, Morent M, Romig T.(Diagnosis of *Sarcocystis* spp. In cattle (*Bos Taurus*) and water buffalo (*Bubalus bubalis*) in Northern Vietnam), 2009, Veterinary parasitology., 166(3-4): 314-320.
15. Li Q Q, Yang Z Q, Zuo Y X, Attwood S W, Chen X W. (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), 2002, Journal of Parasitology., 88(6): 1259-1261.
16. Motamedi Gh R, Dalimi A, Nouri A, Aghaeipour K. (Ultrastructural and molecular studies on fat and thin macrocysts of *Sarcocystis* spp. isolated from naturally infected goats), 2010, Archives of Razi Institute., 65(2): 91-97.

17. Oryan A, Sharifiyazdi H, Khordadmehr M, Larki S. (Characterization of *Sarcocystis fusiformis* based on sequencing and PCR-RFLP in water buffalo (*Bubalus bubalis*) in Iran), 2011, Parasitology Research., 109(6): 1563-1570.
18. Salah A, Abu-Elwafa , Ibrahima E A, Abbas, Mostafa A , Al-Araby A. (Ultrarstrure of *Sarcocystis buffalonis* (Huong *et al*., 1997) infection water buffaloes (*Bubalus bubalis*) from Egypt) , 2015, Intranational Jornal of advanced research, 3(1): 452-457.
19. Saitou N, Nei M. (The neighbor-joining method: A new method for reconstructing phylogenetic trees), 1987, Molecular Biology and Evolution, 4(4): 406-425.
20. Tamura K, Nei M, Kumar S. (Prospects for inferring very large phylogenies by using the neighbor-joining method), 2004, Proceedings of the National Academy of Sciences (USA)., 101(30): 11030-11035.
21. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. (MEGA6: Molecular Evolutionary Genetics Analysis version 6.0), 2013, Molecular Biology and Evolution., 30(12): 2725-2729.
22. Yang Z Q, Zuo Y X .(The new views of the researchers on cyst forming coccidia species including *Sarcocystis* by using the molecular biological techniques), 2000; Chinese Journal of Parasitology and Parasitic Diseases., 18: 120–126.
23. Yang Z Q, Zuo Y X , Ding B , Chen X W, Luo J, Zhang Y P.(Identification of *Sarcocystis hominis*-like(Protozoa: Sarcocystidae) cyst in water buffalo (*Bubalus bubalis*) based on 18s rRNA gene sequences) , 2001, Journal of Parasitology, 87: 934-937.
24. Yang ZQ, Zuo YX, Yao YG, Chen XW, Yang G C, Zhang YP. (Analysis of the 18S rRNA genes of *Sarcocystis* species suggests that the morphologically similar organisms from cattle and water buffalo should be considered the same species), 2001, Mol Biochem Parasitology, 115(2): 283-8.
25. Zhao-Qing Yang, Qing-Qing L, Yang-Xian Z, Xin-Wen C, Yong-Jiu C, Long N, Chang-Gue W, Jia-Shun Z, Attwood S W, Xue-Zheng Z, Ya-Ping Z.(Characterization of *Sarcocystis* species in domestic animals using a PCR-RFLP analysis of variation in the 18S rRNA gene: a cost-effective and simple technique for routine species identification) , 2002, Experimental Parasitology, 102: 212–217.