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ISOLATION AND MOLECULAR IDENTIFICATION OF *SARCOCYSTIS MOULEI* FROM SHEEP AND GOATS (LOCAL BREED) IN BASRAH PROVINCE - IRAQ

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Abstract: *Sarcocystosis* is caused by many forms of *Sarcocystis*. It's a protozoal infection with worldwide distribution in many species of animals. The current study was aimed at screening infection prevalence and molecular identification of the macroscopic and microscopic *Sarcocystis* spp. infecting Iraqi sheep and goats. *Sarcocystis* was examined through meat inspection in a slaughterhouse, followed by molecular identification using PCR, sequencing, and phylogenetic analysis of the 18srRNA gene. Macroscopic cysts of *Sarcocystis* were observed in the esophagus of both sheep and goats by the naked eye. The overall prevalence of infection with *Sarcocystis* spp. was 9.1 % (76 / 830) in sheep and 8 % (12/150) in goats. The overall prevalence of the macroscopic cysts of the *Sarcocystis* spp. was 8.97% (88/980). The prevalence of *Sarcocystis* spp. was higher in females 12% (41/340) than in males 7.1% (35/490) in sheep, as well as, the infection of goats was higher in females 10% (7/70) than in males 6.25% (5/80). The comparison of the obtained sequences of the local tested samples of *S.moulei* with those previously reported for different *Sarcocystis* spp., which were recorded in Genbank, revealed that all the tested specimens were represented for *S.moulei.* Moreover, sequence alignments of the partial 18srRNA gene ascertained the presence of genetic differences, revealing the heterogenic nature of the gene among different strains of the same *Sarcocystis* spp. in relation to the geographic distribution. This is the first study for the evaluation of the PCR technique for genetic identification of the macroscopic *S.moulei* infected in Basrah in southern Iraq and the first reported for the infection of sheep and goats with *S.moulei.* It is also the first recorded in Iraq of six strains of *S.moulei* in sheep and goats.

Keywords: *Sarcocystis moulei*, Sheep , Goats, Basrah, Prevalence.

INTRODUCTION

Sarcocystis species are coccidian intracellular protozoan parasites from the genus *Sarcocystis*, which belongs to the Apicomplexa phylum's family Sarcocystidae. Sarcocystosis is a zoonotic protozoan illness that affects a wide range of mammals, reptiles, and birds worldwide (Fayer *et al.*, 2015).

There are around 200 genuine species of *Sarcocystis*, which range in pathogenicity to the host, ranging from virulent to severe disease. Many species are zoonotic (Dubey, 2015; Dubey *et al.*, 2016).

This parasite requires two hosts to complete their life cycle (prey and predator), which are referred to as intermediate and final hosts, respectively. *Sarcocystis* cysts are classified as either microscopic or macroscopic (Dubey, 2015). The large macrocyst can be seen with the naked eye, and it may reach 3 cm in size accordingly. As the infection progressed over time, the size of these cysts increased (Bunyaratvej *et al.*, 1982), and the small microcyst can only be seen with a microscope(Yang *et al.*, 2001; Fayer, 2004; Barham *et al.*, 2005). PCR tests and sequencing processes are now considered far more feasible, accurate, and reliable than previous approaches based on visual traits. (Gjerde, 2013; Huang *et al.*, 2019).
The current study aimed to screen the prevalence of *Sarcocystis* infection and apply genetic identification of the macroscopic *Sarcocystis* spp. in Iraqi sheep and goats in southern Iraq because several species of *Sarcocystis* are pathogenic and result in severe disease, which can lead to abortion and carcass condemnation in meat-producing animals; also, most species are described as zoonotic.

MATERIAL AND METHODS

Sample collection

A total of 980 esophageal samples, including 830 sheep (490 males and 340 females) and 150 goats (80 males and 70 females) aged 1-6 years, were randomly inspected and collected during postmortem inspections of the slaughtered animals at the slaughterhouse in Basrah city, Iraq. Additionally, entire fresh esophageal samples were collected from animals slaughtered outside the abattoirs of butcher markets. Samples were collected during the period from December 2020 to November 2021. All esophageal tissues were inspected for the detection of macroscopic cysts before taking samples. The samples were stored in sealed plastic bags inside ice-cooled boxes and submitted to the Parasitology Laboratory at the College of Education for Pure Sciences, Biology department, University of Basrah for further microscopic examination and other experiments.

Morphologic identification

Esophageal muscles were examined by the naked eye to detect macroscopic *Sarcocystis* spp. The macroscopically visible *Sarcocystis* spp. They were carefully isolated from muscle samples, and their shape and size were determined. The size and dimensions of the macroscopic Sarcocystis were measured by a ruler (Zangana and Hussein, 2017, Dong *et al.*, 2018).

Molecular identification using 18srRNA

The molecular analysis was used for confirmation of species identification and specificity to an intermediate host for samples kept at -20°C until processed for molecular study (18srRNA gene for PCR teqnique).

DNA extraction

The DNA was extracted from macrosarcocystis of both infected sheep and goats using the DNA extraction Kit® Analytikjena FFBE according to the manufacturer's protocol.

PCR amplification and sequencing

PCR reactions were applied to each DNA extract using the primer sets that were designed according to the 18srRNA sequence of *S. moulei* (Table 1). Both forward and reverse strands of the isolated DNA were shipped to Korea for sequencing. For the goal of looking for particular DNA sections of *Sarcocystis* spp., positive PCR results were directly sequenced using oligonucleotide primers. and were purified with Exo-Sap (Macrogen Inc., South Korea) treatment according to the manufacturer's recommendations. Forward and reverse sequencing reactions were performed using the ABI Prism BigDye terminator cycle sequencing reaction kit, version 1.1 (Macrogen Inc., South Korea). Sequenced fragments were purified by Macrogen Inc. (South Korea) and resolved by capillary electrophoresis using a 3730xl Genetic Analyzer (Macrogen Inc., South Korea). The nucleotide sequences were linked with a single sequence. The resulting sequences were aligned and subjected to phylogenetic analyses of the 18s rRNA genes against previously sequenced *Sarcocystis* spp. as well as with other registered sequences of *Sarcocystis* spp. from intermediate hosts, retrieved from GenBank using the BLAST program(http://www.ncbi.nlm.nih.gov/BLAST) (Rubiola et al., 2018).

Table 1: Oligonucleotide primers sequence target the 18srRNA gene and PCR amplification condition.

***Sarcocystis* spp.**	**Primers(bp)References**	**PCR reaction condition**			
		Step	Temprature^{o}C	Time	**cycles**
S. moulei	Forward – 5´GCACTTGATGAATTCTGGCA 3´	Denaturation	94	5 min	**35**
	Reverse- 5 ´CACCACCCATAGAATCAAG 3´	Denaturation	94	2 min	
		Annealing	60	30sec	
	Siez :(637-bp)	Extension	72	5 min	
	Motamedi *et al* ., 2010 ; Dakhil *et al*., 2017	**Terminal**	**72**	**2 min**	

Statistical analysis

The results were analyzed statistically using the statistical package for social sciences (SPSS). The differences in the prevalence of males and females of sheep and goats were analyzed by the Chi-square test. They are statistically significant at the probability level of $P \leq 0.05$.

RESULTS

Morphologic Identification

The results showed the macroscopic *Sarcocystis* spp. appeared as a cyst on the surface of the esophageal wall on the serous membrane or deeper in the muscle layer in different sizes ranging from (5 x 2.5-15 x 7 mm) and appeared white in color and various shapes like oval, round in sheep as shown in Figure 1 (A, B, and C) and goats as shown in Figure 2 (A, B, and C).

Prevalence of Sarcocystosis in sheep and goats in Basrah governorate

Macroscopic *Sarcocystis* cysts were observed in the esophagus of both sheep and goats by the naked eye. Table 2 shows the overall prevalence of *Sarcocystis* spp. infection was 9.1% (76/830) in sheep and 8% (12/150) in goats. The overall prevalence of sarcocystosis was higher in two-animal sheep and goats than in males, 7.01% (40/570), while in females, 11.70 % (48/410) in two-animal sheep and goats. The overall prevalence of the macroscopic cysts of the *Sarcocystis* spp. was 8.97% (88/980) in the second and third tables. The prevalence of *Sarcocystis* spp. was higher in females 12% (41/340) than in males 7.1% (35/490) in sheep, as well as in goats 10% (7/70) than in males 6.25% (5/80), Table (3).

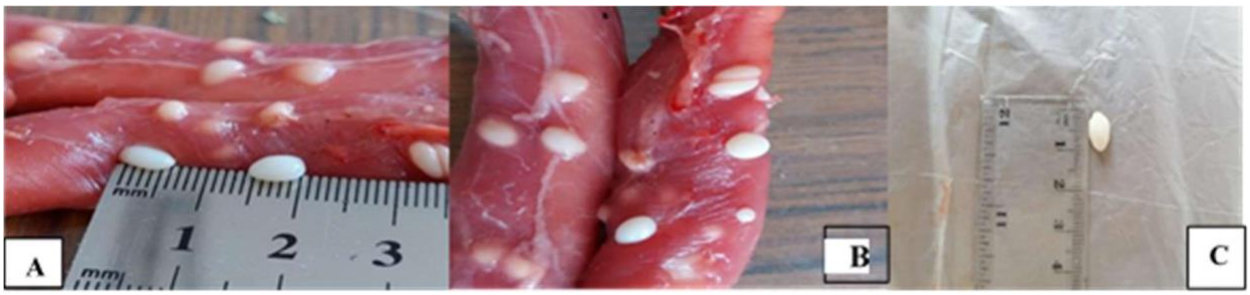

Figure 1: Gross appearance of macroscopic *Sarcocystis* spp. in the esophagus of the inspected animals, A,B and C of heavily infected sheep .

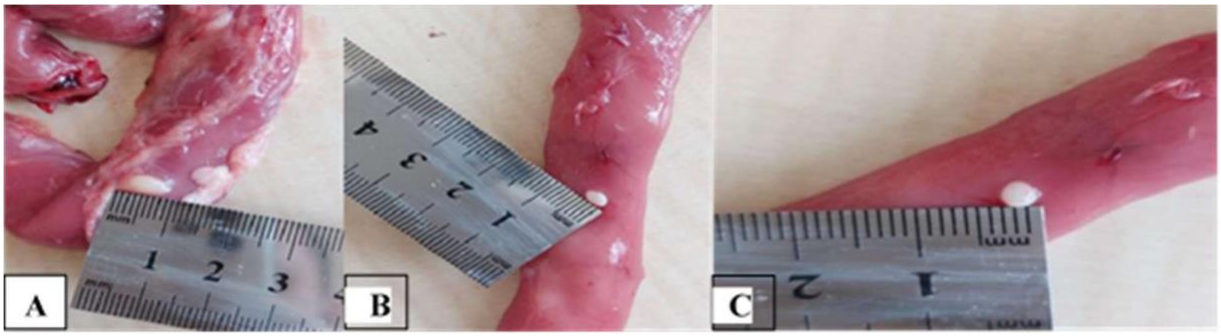

Figure 2: Gross appearance of macroscopic *Sarcocystis* spp. in the esophagus of the inspected animals, A,B and C of heavily infected goats .

Table 2: Prevalence of *Sarcocystis* spp. in esophagus to the sheep and goats.

Animals	examined	infected	infected (%)
Sheep	830	76	**9.1**
Goats	150	12	**8**
Total	**980**	**88**	**8.97**

Table 3: Prevalence of *Sarcocystis* spp. among males and females of sheep and goats

Sex	Sheep		Goats		Total Number (examine) in two animal	Total Number (infected) in two animal
	No. examined	No. infected	No. examined	No. infected		
Males	490 (59.03%)	35 (7.1%)	80 (53.3%)	5 (6.25%)	570	**40 (7.01%)**
Females	340 (40 %)	41 (12 %)	70 (46.6%)	7 (10%)	410	**48 (11.70%)**
Total	**830**	**76 (9.15%)**	**150**	**12 (8%)**	**980**	**88 (8.97%)**

Molecular Study

Fifty esophageal macroscopic muscle cysts of sheep and fifty esophageal macroscopic muscle cysts of goats were used for PCR amplification by using the partial 18srRNA gene. The PCR amplification showed that all isolates have a positive range on gel electrophoresis. The partial 18s rRNA gene was amplified in all positive samples and produced the expected size of the PCR amplicon of 637 bp in both types of animals (Fig. 3).

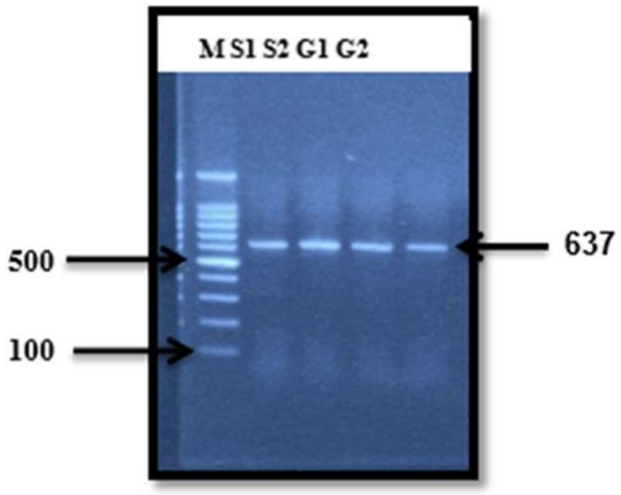

Figure 3: PCR products of partial 18s rRNA gene of *S. moulei* appears in sheep & goats , presented bands at 637 bp on 1% agarose gel electrophoresis. M: 100 bp DNA Ladder. Lanes are Positive to the sheep (S1 and S2) while Lanes are Positive to the goats (G1 and G2) replicate.

Sequencing analysis of 18srRNA gene and phylogenic tree:

The six sequences of *S. moulei* were placed within the tree (Sample 1, Sample 2, Sample 3, Sample4, Sample 5, and Sample 6) were found in all animals (sheep and goats) diagnosed was *S.moulei* and compared with the identity of BLAST in NCBI, which confirmed the analysis of the DNA sequences of the 18srRNA gene using advanced primers and the production of a piece containing the nucleotide sequences based on the Basic Local Alignment Search tool (BLAST) as shown in figure (4). The phylogenetic analysis compared with *Sarcocystis* 18srRNA sequences from previous sequences registered in the GenBank database. The results of the genetic tree were shown (Fig. 5) using the 18srRNA gene of the species of the genus *Sarcocystis* as it showed that all isolates of the genus *Sarcocystis* when they are identified with Gen Bank, The five study samples lined up in one group, where sample 1 and 2 are closely related, and samples (3,4,5) are the closest, respectively, while the presence of a strain of type *S.moulei* strain Ecy1 in sheep (sample 6) located in Basrah Governorate, and that this strain has previously Gen Bank deposited sequence with accession number KF489430.1 from Iran sheep percentage was 92%.

As for the other samples, the genetic tree showed its kinship with the sheep of Iran KF489423.1, KF489430.1 and Australia by 92.61% for sample 1, 92.77% for sample 2, 90.89% for sample No 3, 93.33% for sample No 4 and 93.23% for sample 5. As shown in figure (5),the study samples are considered the first recorded of *S. moulei* parasite in sheep and goats in Iraq, especially in Basrah governorate.

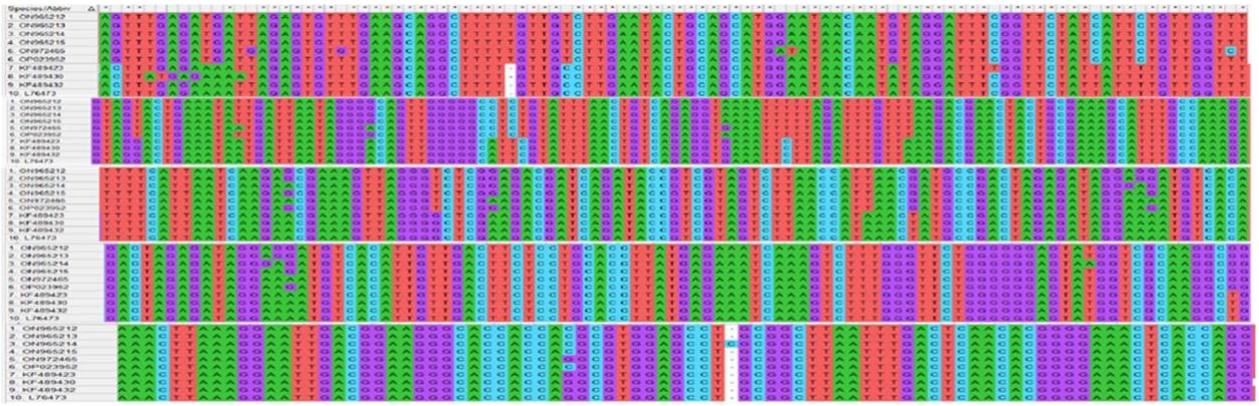

Figure 4: Sequence alignment of the current study samples with the sequence of samples of similar studies

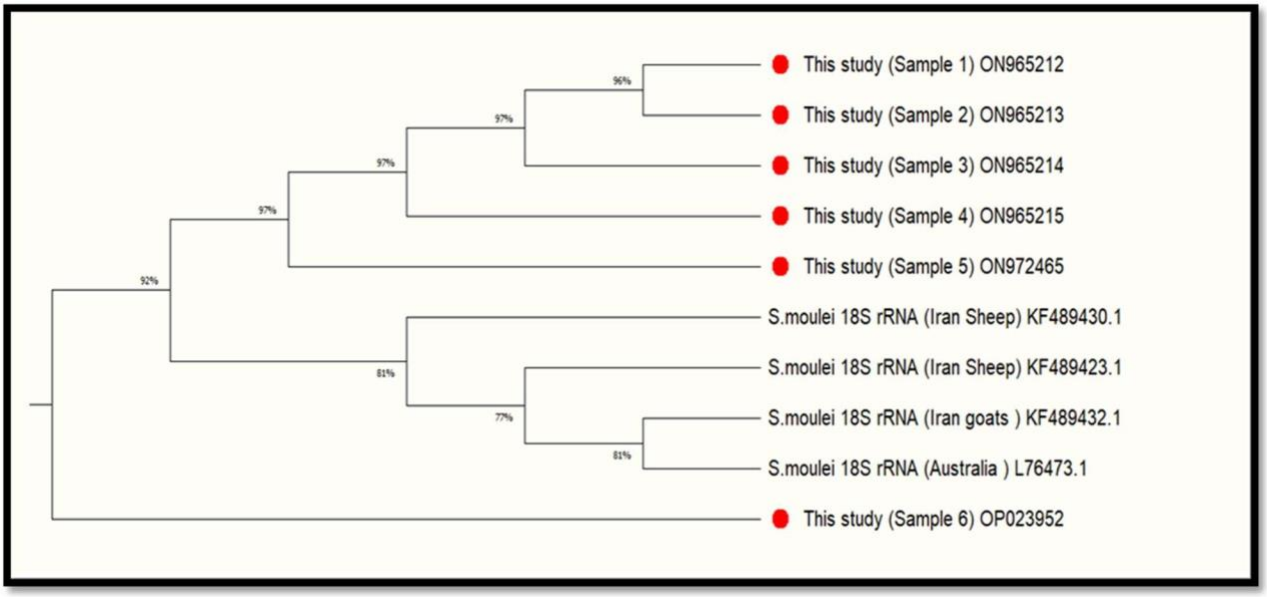

Figure 5: Phylogenetic tree of selected *Sarcocystis moulei* by the program MEGA11 using maximum likelihood-based on 18srRNA gene sequences. The strain are represent by Sample1(ON965212),Sample2(ON965213),Sample3(ON965214),Sample4(ON965215),Sample5 (ON972465(and Sample 6 (OP023952) . Neighbour-joining method was used to create a guide tree as pairwise and multiple sequences alignments. Sanger sequencing technique was used for sequencing purpose .

DISCUSSION

The current study is the first record in Iraq of six strains of *Sarcocystis moulei* in sheep and goats. In the current study, the muscles of the esophagus were examined previous studies indicate that this organ is the most common site for *Sarcocystis* infection (Latif *et al.,* 1999; Barham *et al.,* 2005;

Beyazit *et al.,* 2007; Al-Hyali *et al.,* 2011; Al Quraishy *et al.,* 2014; El- Morsey *et al.,* 2019; Minuzzi *et al.,* 2019; Gjerde *et al.,* 2020).

The infection of macroscopic *Sarcocystis* that was recorded in the present study agrees with the results of Swar and Shnawa, (2021), in slaughtered sheep and goats in Erbil, northern Iraq, was 9.5% in sheep and (8.8%) in goats.

The results of macroscopic *Sarcocystis* show that the prevalence of infections is higher than recorded by Latif *et al.* (1999) in Baghdad Province and Hussein (2015) in Duhok Province, which is 1.2% and 2.6% in sheep and goats, respectively. While the prevalence of infection was lower than the infection reported by Barham *et al.* (2005) in Sulaimanyia province, which is 33.6% in goats.

The infection of *Sarcocystis* was less than recorded in several studies in the world, including Beyazit *et al.* (2007) in Turkey, which showed that 24.5% of sheep were infected with macrocysts of *S.gigantea* and 86.5% of them were infected with the microscopic *Sarcocystis* of *S. ovicanis*, and Abo-Shehada (1996) in Jordan, which found 11.3% of sheep and 11.7% of goats over one-year-old were infected. Additionally, the prevalence of sarcocystosis in this study agrees with Minuzzi *et al.,* (2019) in southern Brazil. Infected of 7.7% with *S.gigantea* macrocysts were recorded in sheep, while the infection was higher in prevalence than recorded by Mirzaei and Rezaei (2016) in Iran.

Furthermore, the source of animals may contribute to the high prevalence of sarcocystosis recorded in Iraq. Most sheep are imported from neighboring countries, and they could be infected from their original sources and transported to the country until slaughtered (Swar and Shnawa, 2021).

The results of the current study indicated that the prevalence of *S.moulei* was slightly higher in females than males in both sheep and goats, with no significant differences. This finding agrees with the results of Swar and Shnawa (2021) and was slightly higher in females (9.2%) than in males 8.9% in both sheep and goats. Also, this finding agrees with the results of Farhang-pajuh et al. (2014), who indicated that the overall prevalence of *Sarcocystis* spp. in sheep was 36.83% (235/638), in males showed a lower rate 7.63%, (38/498) and was less infected than females 35%, (49/140). Moreover, the present results are in line with Zangana and Hussein (2017) and Metwally et al. (2019) found no significant differences between males and females of both sheep and goats.

The molecular study relied on the 18srRNA gene using PCR technology and sequencing analysis. The nitrogen base rules were dependent on distinguishing between strains of *S.moulei* in sheep and goats because of the variable regions of the 18s rRNA gene acting as useful targets for the classification and characterization of dissimilar species. (Neefs *et al.,* 1991; Yang *et al.,* 2001; Dakhil *et al.,* 2017). Moreover, 18srRNA is very convenient for phylogenetic investigations due to its high conservation, and the examination of its variable regions permits the identification of species within a genus (Maidak *et al.,* 1997; Ng *et al.,* 2015). Also, the 18s rRNA gene has been widely used as an appropriate diagnostic target for the exact study of related species of *Sarcocystis* spp. The evolution of genetically stable conservative regions present in the 18srRNA gene, which

aids in the design of primers that can be used to amplify the same gene in related species (Holmdahl *et al.*, 1999; Li *et al.*, 2002; Dahlgren & Gjerde, 2007; Jehle *et al.*, 2009) and the evolution of genetically stable conservative regions present in the 18srRNA gene, which aids in The mosaic structure of this gene provides flexibility in experimental designs for various phylogenetic studies (Olsen and Woese, 1993).

Yang *et al.* (2001) showed that the similarities morphologically between the cysts taken from sheep, goats, and cows do not distinguish between the types of this genus. As indicated by Motamedi *et al.* (2010), the different forms of the two large parasite sacs in goats in Iran are the thin and full sacs isolated from different parts of the animal body, but by PCR technique based on the 18srRNA gene proved that both belong to the same species, *S.moulei*, and this is consistent with the current study.

The PCR amplification of fifty macroscopic *Sarcocystis* revealed that all isolates have positive bands on gel electrophoresis. The partial 18srRNA gene was amplified in all positive samples and produced the expected amplicon PCR size of 637 bp for both animals.

The current phylogenetic analysis was successfully inferred from near full-length 18srRNA sequences and compared with previous sequences presented in GenBank. The occurrence of cross-infection may happen as seen in samples 1–5 belonging to *S. moulei* in both sheep and goats in identified accession numbers KF489423.1(Iran sheep) and KF489430.1 (Iran sheep). While (Sample 6) *S. moulei* strain Ecy1 was only found in sheep, it is a new species that differs from the other five strains that were recorded, and this explains the deviation of Sample (6) from the other five samples in the genetic tree. This finding agrees with the results of Motamedi *et al.* (2010) whorecorded this strain in sheep only.

CONCLUSION :

The result concludes that the current study is the first record in Iraq of six strains of *S. moulei* in sheep and goats. In addition, the findings of this study provide useful information for veterinarians and researchers regarding the epidemiology of *Sarcocystis* spp. infecting sheep and goats in our area of Basrah, southern Iraq.

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