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MOLECULAR ANALYSIS AND PHYLOGENIC TREE OF *SARCOCYSTIS* SPECIES ISOLATED FROM SHEEP IN ERBIL PROVINCE-KURDISTAN REGION/ IRAQ

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ABSTRACT

BACKGROUND: A group of cyst-forming parasites known as *Sarcocystis* species infect both people and animals worldwide. Some of these species affect the host with clinical and subclinical illnesses that can cause financial losses. The diversity and importance of *Sarcocystis* spp. are poorly studied.

METHODS: One hundred ninety tissue sheep samples (esophagus and tongue) were collected from the new Erbil slaughterhouse. The PCR method was used to amplify the *18S ribosomal RNA* gene to differentiate *Sarcocystis* spp.

RESULTS: Out of 190 sheep that were examined macroscopically and microscopically using tissue squash preparation, 36.32% were infected with *Sarcocystis* spp. in Erbil Province. Statistically, there was no significant difference between sexes and the highest rates were found among 1-3 years 40 (57.97%), and the lowest rate of infection was revealed in the less than one year 12 (17.39%) age group. The sequence comparisons showed the highest similarity (99.6-100%) between *S. tenella* (ON645286-ON645288) obtained from sheep in Erbil Province and *S. tenella* from Egypt (MG515220) and Iran (MT565493 and KF489419) due to nucleotide changes in the sequences alignments.

CONCLUSION: Multiple alignments revealed some differences between the consensus sequences of the isolates identified in this investigation and those of previously published isolates. A phylogenetic analysis of the *18S rRNA* gene was used to find new *Sarcocystis* spp.

***KEYWORDS*:** Epidemiology, *Sarcocystis* species, Molecular identification

1- INTRODUCTION

Protozoan species belong to the genus Sarcocystis and are found in mammals, birds, and reptiles. This parasite is characterized by two hosts and a prey-predator life cycle. Sporocysts mostly develop in the small intestine of the definitive host, whereas Sarcocysts primarily form in the muscles of the intermediate host (Gjerde, De la Fuente, Alunda, & Luzón, 2020). The definitive host obtains the infection mostly by eating muscle tissues containing mature *Sarcocysts*, while it is transmitted to the intermediate host through contaminated food and water (Marandykina-Prakienė et al., 2022). Carnivores and omnivores both act as final and intermediate hosts in the life cycle of this parasite (Alhayali, Hasan, & Al-Mallah, 2020). Sheep are one of the most commonly infected hosts among livestock animals, with a global distribution. *Sarcocystis gigantea* and *S. medusiformis* are two nonpathogenic species that have been found in sheep and are known to create macroscopic cysts. Cats are also the definitive hosts for these parasites. Canids are the primary hosts for some species, such as *S. tenella* (syn. *S. ovicanis*) (Zainalabidin, 2021) and *S. arieticanis*, which cause microscopic cysts and should be deemed harmful. Different phenotypic characteristics, like structure, size, thickness of the cyst wall, (Bittencourt et al., 2016) and protrusion-villar arrangement, allow different *Sarcocystis* spp. (Pestechian et al., 2021). Depending on the species, *Sarcocystis* has different levels of pathogenicity. The inspected sheep meat from Iraqi abattoirs, which is frequently found to be extensively contaminated with macrocysts, will be deemed unfit for human consumption. A further challenge is that most studies on ovine *Sarcocystis* infection are limited to only examining the carcasses at the slaughterhouse without focusing on identifying the exact species of *Sarcocystis*, which may return harmless and fit for human consumption and thus reduce the economic losses (Al-Saadi, Al-Mussawi, &

Muhammed, 2020; Dessì et al., 2022). Recently, molecular techniques have been used as an epidemiological and diagnostic tool to identify *Sarcocystis* spp. responsible for an infection (Prakas, Rehbein, Rudaitytė-Lukošienė, & Butkauskas, 2021). The use of the appropriate markers during molecular characterization has become essential for accurately identifying *Sarcocystis* spp. and investigations into the genetic background of these parasites (El-Morsey, Abdo, Zaid, & Sorour, 2021). For comparative molecular investigations on *Sarcocystis* spp. in cervids, nucleotide sequences of the mitochondrial *COX1* gene and/or nuclear *18S ribosomal RNA* gene (*18S rRNA*) of about 30 species are currently available (Metwally, Al-Damigh, Al-Turaiki, & El-Khadragy, 2019). The aim of the study is to recognize different *Sarcocystis* spp. using *18S rRNA* genes that were isolated from naturally infected sheep in Erbil Province.

2- METHODOLOGY

The current study, which started from May 2022 to February 2023, ensure that all of the sheep were in good health before being slaughtered, and careful examination was done before the slaughtering. The gender of each sheep was recorded, and the sheep's age was estimated based on a dental examination that counted the number of newly erupted permanent incisors as well as the number of temporary teeth (Greenfield & Arnold, 2008).

2.1- Sample Collection:

At the new Erbil slaughterhouse, the entire esophagus and tongue were removed during postmortem examinations, and the tissues were subsequently transported in boxes with ice packs to the laboratory.

2.2- Macroscopic Examination:

On the same day as the collection of the tissue, a macroscopic analysis was completed. The internal and external walls of the whole tongue and esophagus were examined macroscopically after being longitudinally sectioned to reveal its lumen.

2.3- Microscopic Examination:

A squash preparation was used for the microscopic examination of fresh tissues for cysts. After placing the individual pieces (2*2 thickness) of each infected tissue on a glass slide and covering them with a coverslip, the sample was crushed as much as possible with the thumb, then the upper glass slide was removed, and the sample was examined at 40X (Al-Saadi et al., 2020).

2.4- DNA extraction and PCR amplification:

The genomic DNA was extracted from each tissue samples (homogenate or purified Bradyzoites) according to the instructions on the Genomic DNA Extraction Kit (Bio-Tech, Korea). The extracted DNA was stored at -20°C, and the following primers were used in a PCR reaction to amplify a 600 bp segment of the *18S ribosomal DNA* gene: forward (Sarco F: 5′-GCACTTGATGAATTCTGGCA-3′) and reverse (Sarco R: 5′-CACCACCCATAGAATCAAG-3′) (Bahari, Salehi, Seyedabadi, & Mohammadi, 2014; Maky & Mohammed, 2021). The conventional PCR was employed using a reaction volume of 30µl for each amplification (12.5µl of PCR Mater Mix, 2µl of each primer (Sarco F and Sarco R) of 10 Pmol, 5µl of DNA template and it was completed by distilled water (8.5 µl). The PCR program has the following settings: one cycle at 95 ^{0}C (5 minutes), 35 cycles at 94 ^{0}C for 30 seconds, 55 ^{0}C (45 seconds), 72 ^{0}C (50 seconds), and a final extension of 1 cycle at 72 ^{0}C for 10 minutes, using a thermocycler machine (Bioer XP, China). Deionized water was used as a negative control in each PCR assay, and electrophoresis on a 1.5% agarose gel was used to see the PCR results. A 5 µl of the PCR products were added to the gel, and the size of the amplicons were determined using a 100-bp gene marker ladder (Gene Direx-USA).

2.5- Nucleotide sequencing and data analysis:

The positive PCR product was commercially sequenced in both directions (Ruibiotech, Beijing, China), and the acquired nucleotide sequences were aligned with reference sequences on the National Center for Biotechnology Information (https://blast.ncbi.nlm.nih.gov/Blast.egi). The nucleotide alignment employed for DNA sequence queries by ClustalW (http://www.ebi.ac.uk/clustalw) (Thompson, Gibson, Plewniak, Jeanmougin, & Higgins, 1997) and the unit matrix options of Bioedit (Hall, 1999), respectively. Through the use of the BLAST algorithm, the partial genes of *Sarcocystis tenella* were saved in the GenBank database under the accession numbers ON645285, ON645286, ON645287 and ON645288. The MEGA7 software's MUSCLE algorithm (Hassan, 2021; Kumar, Stecher, & Tamura, 2016) was used to create multiple sequence alignments, which were then utilized to

choose a nucleotide substitution model and create phylogenetic trees using the maximum likelihood approach.

2.6- Statistical Analysis:

Scatter plot graphing software (GraphPad Prism v.7, CA, USA) was used for data analyses to define the incidence rates of *Sarcocystis* spp. in sheep.

3- RESULT AND DISCUSSION

Out of 190 sheep that were examined macroscopically and microscopically using tissue squash preparation, 36.32% were infected with *Sarcocystis* spp. as shown in Table 1. The result, agreed with (Nasr, Hussen, & Mekawy, 2013) revealed that the prevalence rate of *Sarcocystis* spp. in ovine was 47.50%, and (Mahran, 2009) stated that, the prevalence rate of ovine *Sarcocystis* was found to be 41.26%. However, the results did not correspond with (Atashparvar, Soukhtezari, & Amir Asalani, 2001), which claim that 6.67% of sheep in Khoram-Abad had *Sarcocystis*. This variation in the prevalence of *Sarcocystis* infection may be caused by the various populations of stray dogs, which are important sources of *Sarcocystis* infection. The highest infection rate was recorded in the oesophagus at 54 (28.42%), and the lower rate was recorded in the tongue at 15 (7.89%). The result agreed with (Metwally et al., 2019) which demonstrated that, an investigation of random muscles samples obtained from 314 slaughtered sheep and goats for the presence of *Sarcocystis* spp., the highest esophagus organ infection rate was 33(25.98%) as compared with the tongue 29 (22.83%) by macroscopic and microscopic examination. Furthermore, (Mirzaei & Rezaei, 2016) showed that oesophageal infection 24(33.2%) was more common as compared with diaphragm muscles 8(9.9%). The distribution of *Sarcocystis* cysts differed between sheep breeds and organs, and the oesophagus was the most frequently infected sheep organ (Hu et al., 2017). Statistically, there was no significant difference between sexes (P = 0.7429), and the highest rate of infection was revealed in males 51 (73.91%) as compared with females 18 (26.09%). The result agreed with (Zangana & Hussein, 2017) which reported that, the overall (79 sheep and 48 goats) prevalence of sarcocystosis was 97.5% in sheep and 100% in goats which recorded and there are no significant difference ($p>0.05$) was observed in infection rate between male of sheep 95.8% and male of goats100% and female of sheep and of goats 100%. These variations may be due to differences in the management conditions in such localities.

Table (1): -The frequency rate of *Sarcocystis* infection in sheep according to the gender

Organs	No. of examined sheep	Total Positive No.	(%)	Gender				P Value
				Male	(%)	Female	(%)	
esophagus	135	54	28.42	39	56.52	15	21.74	0.7429
Tongue	55	15	7.89	12	17.39	3	4.35	
Total	190	69	36.32	51	73.91	18	26.09	

Table 2 expressed that, the rate of infection increased proportionately with the animal's age. The highest rates were found among 1-3 years old 40 (57.97%), and the lowest rate of infection was revealed in the less than one year 12 (17.39%) age group. These differences were not statistically significant (p=0.9148). The result agreed with (Nasr et al., 2013) who reported older sheep 34 (56.67%) have a higher infection rate than younger sheep 23 (38.33%). These variations might result from variations in sample size, management practices, and geographic location. Furthermore, (Elmishmishy, Al-Araby, Abbas, & Abu-Elwafa, 2018; Zainalabidin, 2021) represented that, small ruminants can acquire the parasite through accidental consumption of the sporocyst, which is the infective stage, from contaminated food and drinking water. Good farm management practices, such as regularly providing clean feed and water trays, will help to decrease exposure to sporocysts and prevent the disease from occurring on the farm. The sporozoites were released when the sporocyst was consumed.

Table (2): -The frequency rates of *Sarcocystis* in sheep according to the age groups

Organs	Total Positive No.	Ages						P value
		<1 years	(%)	1-3 years	(%)	>3 years	(%)	
Esophagus	54	9	13.04	32	46.38	13	18.84	0.9148
Tongue	15	3	4.35	8	11.59	4	5.80	
Total	69	12	17.39	40	57.97	17	24.64	

The extracted DNA of *Sarcocystis* spp. was amplified using the partial *18S rRNA* gene at the expected product size (~600bp) of all 69 samples. Twenty-five PCR products (15 esophagus and 10 tongues) were successfully sequenced in both directions, and the results indicated that all samples belonged to *S. tenella*. The consensus genomes of the isolates acquired in the current investigation revealed some variations when compared with MG515220 (Elmishmishy et al., 2018), MT565493 (Salehi, Spotin, Rostamian, & Adami, 2022) and KF489419 (Bahari et al., 2014). Overall, 14 (58%) isolates under the accession number of ON645285 out of 25 samples had a similarity coverage of 100% to *S. tenella* (MG515220, MT565493, and KF489419) and 5 (20%) isolates (ON645286) showed 99.80% identity to *S. tenella* due to nucleotide changes (T→G) at position 73. Furthermore, 4 (16%) isolates (ON645287) had 99.80% similarity coverage to *S. tenella* due to a change in nucleotide (T→C) at position 100. On the other hand, in two of the isolated sheep (ON645288), the change occurred at positions 73 and 391 with T→G and G→A as compared with *S. tenella* as shown in Table 3 and Figure 1. The result agreed with (Bahari et al., 2014; Pestechian et al., 2021), which stated that *S. gigantean* and *S. tenella* were the only species of Sarcocystis that had been found by molecular analysis. As well, (Rahdar & Kardooni, 2017) reported that the nucleotide sequence was compared to other nucleotides in NCBI, and the results showed that they have 100% similarity with *S. tenella* and *S. capricanis*. Previous reports throughout the world reported *S. tenella* infection in sheep from Tunisia (Amairia et al., 2018) and Iraq (Whaeeb & Khalaf, 2016) is one of the pathogenic species that might cause cysts and the number of eaten sporocysts (Bahari et al., 2014) and the host's immune system (Al-Saadi et al., 2020) determine the severity of the clinical symptoms brought on by this species (Zhu et al., 2022).

Table (3): -Genotyping *S. tenella* in sheep identified by *18S rRNA gene* in Erbil Province

Our sequences (No. of samples)	NCBI sequences	Position of Polymorphism in partial sequences	nucleotide change	Percentage of Polymorphism	Percentage (%)
ON645285 (14/25)	MG515220, MT565493 and KF489419	No Polymorphism in partial sequences	nil	100%	56
ON645286 (5/25)	MG515220, MT565493 and KF489419	73	T→G	99.80%	20
ON645287 (4/25)	MG515220, MT565493 and KF489419	100	T→C	99.80%	16
ON645288 (2/25)	MG515220, MT565493 and KF489419	73 and 391	T→G and G→A	99.60%	8

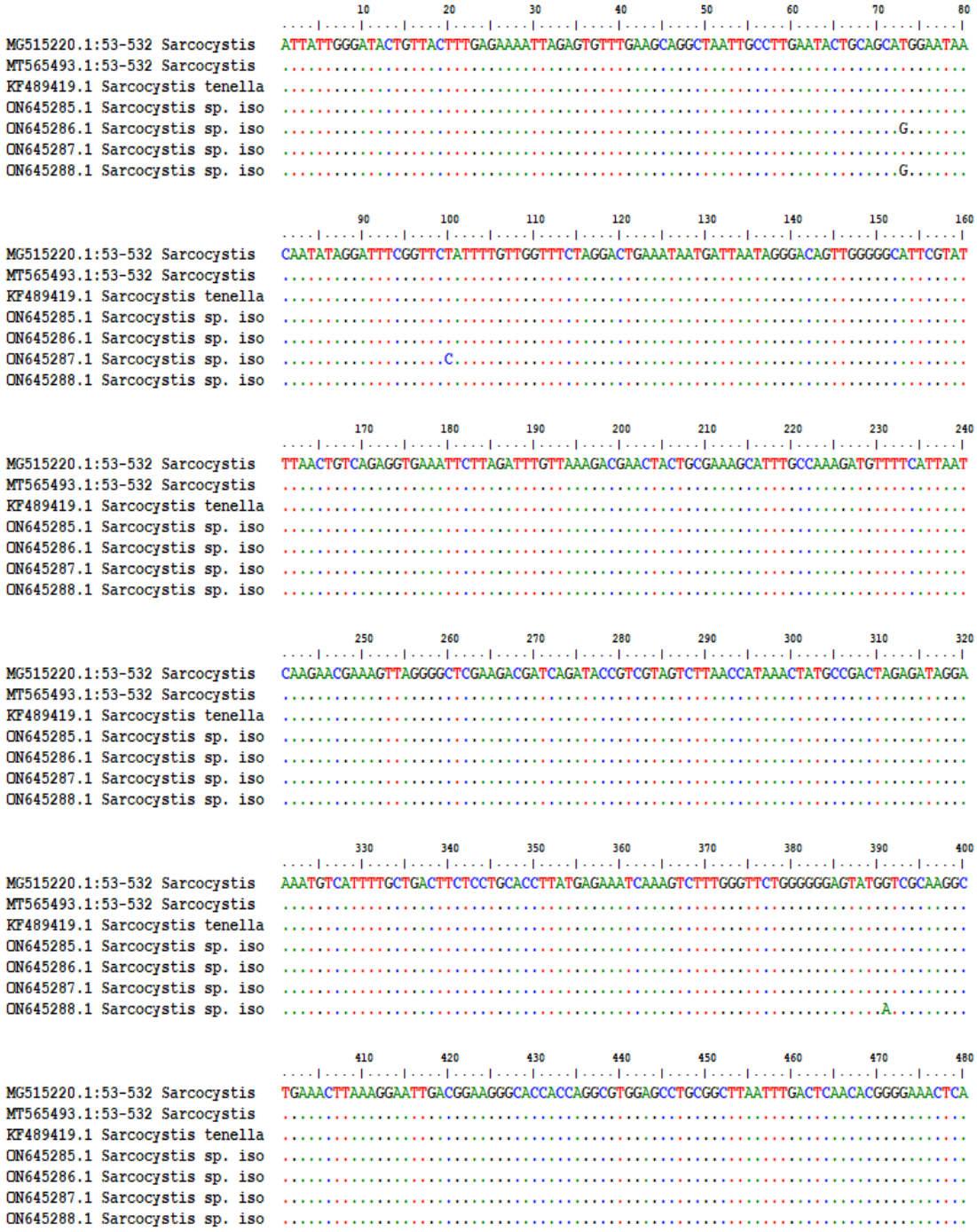

Fig.(1): -Multiple sequence alignment of the partial *18S rRNA gene* of *S. tenella* isolated from slaughtered sheep and previously GenBank-deposited *S. tenella* strains resulted

The maximum likelihood approach was used to create a phylogram based on *18S rRNA* gene sequences. Phylogenetic relationships revealed that, all *S. tenella* haplotypes had been clustered in a single clade, with KF489419, MT565493, MT498780, MT445218, (MG515213-MG515221), MH236177, MH191112, LC364052, (LC364049-LC364050), MF039329, LC214880, MT560372, MN994621, MT757937, MT757931, MT560372, and MT569891 isolates sharing a high degree of closeness as shown in figure 2. The result agreed with (Dong et al., 2018; Elmishmishy et al., 2018) revealed that low nucleotide and haplotype diversities of *S. tenella* may be explained by their rigorous circulation between sheep and the end host (cats or dogs), providing few opportunities for mutations caused by the transmission of the parasite among variable hosts . As well, (Swar & Shnawa, 2020) explained that the variable

portions of the *18S rRNA* gene act as important targets for the classification and characterization of diverse species (Ng, Fong, Subramaniam, Shahari, & Lau, 2015). Additionally, *18S rDNA* is highly useful for phylogenetic studies within a genus (Gjerde, 2013).

Fig. (2): - Phylogenic tree of *S. tenella* isolated from sheep which deposited in genBank

4- CONCLUSION

This study provided the most comprehensive record of *Sarcocystis* in Erbil Province, and sheep could be considered as an alternative intermediate host for *S. tenella*. As well, complete sequence analysis would be helpful to gain a better understanding of the genetic diversity among *Sarcocystis* spp. isolated around the globe.

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6- DECLARATIONS:

Our manuscript entitled "**MOLECULAR ANALYSIS AND PHYLOGENIC TREE OF *SARCOCYSTIS SPECIES* ISOLATED FROM SHEEP IN ERBIL PROVINCE**" is original, does not infringe any copyright or other proprietary rights of third parties, is not submitted for publication to another journal, and has not been previously published.

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