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Histopathology and molecular identification of *Sarcocystis* species forming macrocysts in slaughtered sheep and goats of Duhok, Iraq

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Abstract

This study aimed to identify *Sarcocystis* species isolated from macroscopic sarcocysts of naturally infected sheep and goats using histopathological and molecular studies. A total of 260 macrosarcocyst samples were randomly collected from 1,337 infected sheep and goats slaughtered at different abattoirs in Duhok province, Iraq, from May 2021 to June 2022. The macroscopic cysts, which were found in the esophagus, diaphragm, and abdominal muscles, were classified into fat and thin cysts. Histopathological examination of the observed fat and thin cysts showed a thick eosinophilic wall, several internal septa-forming compartments enclosing numerous bradyzoites, and mild mononuclear inflammatory cells infiltrating around the cysts. The *18 Subunit ribosomal RNA (18S rRNA)* and *28 Subunit ribosomal RNA (28S rRNA)* genes of *Sarcocystis* spp. were amplified by polymerase chain reaction (PCR) from 200 macrosarcocysts samples. Molecularly, the DNA sequencing results obtained from fat macrocysts of sheep and goats were found to be identical to *Sarcocystis gigantea*, and from thin cysts of sheep proved to be similar to *Sarcocystis medusiformis*, while from thin macrocysts of goats were found to be identical to *Sarcocystis moulei*. Alignment and phylogenetic analysis observed a very close relationship between identified species of *Sarcocystis* and other *Sarcocystis* DNA sequences of sheep and goats across the world. To our knowledge, this is the first histopathological and molecular study for identification of *Sarcocystis* spp. isolated from different macroscopic forms of sarcocysts of sheep and goats in Iraq.

Introduction

Sarcocystis (phylum; Apicomplexa) is an intracellular cyst-forming protozoan parasite that infects a wide range of vertebrates, livestock, and humans with more than 190 available species.[1] The life cycle of *Sarcocystis* spp. require two obligatory hosts: an intermediate host, in which merogony and cyst formation develop in different muscles (muscular sarcocystosis) of herbivorous or omnivorous, and a definitive host, in which sporogony and gametogony develop in the intestinal lumen (intestinal sarcocystosis) of carnivorous.[2] *Sarcocystis* usually develops macroscopic cysts up to 1.00 cm in length in muscle of the esophagus, larynx, tongue, diaphragm, abdomen and the rest of the carcass. Macrosarcocyst is a mildly pathogenic form of the parasitic species; therefore, it is regarded as a chronic infection with subclinical signs.[3]

There are several *Sarcocystis* spp. forming macroscopic cysts that are distributed in nature and have been recorded worldwide, such as *Sarcocystis gigantea* (*S. ovifelis*) and *S. medusiformis* in sheep, *S. moulei* (*S. caprafelis*) in goats, *S. fusiformis* and *S. buffalonis* in water buffalo, *S. hirsute (S. bovifelis)* in cattle, and *S. ileyi* in ducks.[4-6] These species are commonly known to cause economic losses in the meat production industry worldwide.[3] This is because meat from sheep and goats severely affected by macro-sarcocysts is often rejected by slaughterhouses as it is unfit for human consumption.[7,8]

In Iraq, the prevalence of macroscopic cysts in slaughtered sheep and goats has varied through visual examination from one province to another. In Baghdad, the prevalence of macrocysts was 4.10% in sheep and 33.60% in goats.[9] While in Erbil, it was 9.50% in sheep and 8.80% in goats.[10] In Duhok, the prevalence of the parasitic infection was 22.40% in sheep and 16.80% in goats.[11] In Wasit and Sulaimanyia provinces, it was 14.44% and 34.00% in goats, respectively.[12,13] In different provinces of Türkiye, the percentage rate of macroscopic cysts in sheep

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has ranged from 6.10 to 66.00%.[14] Furthermore, in various abattoirs in Iran, the percentage rates of slaughtered sheep were 66.00, 57.70, 33.30, and 18.63%.[15-18]

Almost all investigations into detection of *Sarcocystis* infection in sheep and goats are limited to slaughterhouses through inspection only, without any attention being paid to the identification of *Sarcocystis* spp. involved.[7,9] Globally, *Sarcocystis* spp. have most commonly been identified by molecular studies using *18 Subunit ribosomal RNA (18S rRNA)*, *28 Subunit ribosomal RNA (28S rRNA)*, *cytochrome c oxidase subunit 1 mitochondrial (cox1)* and *internal transcribed spacer 1 (ITS-1)* as the most reliable and the most effective gene markers.[19-21] Although the *mitochondrial cox1* and *ITS-1* sequences are considered precise molecular markers for Sarcocystidae, *18S rRNA* gene has widely been used to differentiate apicomplexan as well as the genus *Sarcocystis* from other eukaryotic species because of its hypervariable regions that interspersed within highly conserved DNA sequences, which makes it a valuable diagnostic marker.[22] Additionally, the use of the *28S rRNA* gene alongside the *18S rRNA* gene has been shown to enhance the investigation of *Sarcocystis* spp.[23]

In Iraq, only two studies have been conducted on macroscopic sarcocysts in the esophagus of infected sheep and goats by molecular analysis targeting *18S rRNA* gene in Karbala and Erbil.[8,10] However, histopathological investigation and molecular analysis regarding sequencing and phylogenic relationships using *18S rRNA* and *28S rRNA* molecular genes for detection and identification of *Sarcocystis* spp. forming macroscopic sarcocysts in the esophagus, diaphragm and abdominal muscles of sheep and goats have not been studied in Iraq. Thus, this histopathological and molecular study was designed to identify *Sarcocystis* spp. in slaughtered sheep and goats in Iraq.

Materials and Methods

Sample collection. During this study from May 2021 to June 2022, a total of 260 macrosarcocysts samples (sample/animal) were randomly collected from 955 infected sheep and 382 infected goats out of 8,500 slaughtered animals from four slaughterhouses (Duhok, Sumail, Zakho, and Amediye) of Duhok province, in Kurdistan region of Iraq. All of the animals were found to be healthy upon antemortem examinations. The age (3 - 7 years old) of the animals were estimated based on the eruption of the permanent incisor teeth before slaughter.[24] The collected tissue samples were then divided into two groups based on the types of animals affected (Table 1).

Macroscopic examination. The esophagus, diaphragm and abdominal muscles of the slaughtered sheep and goats were examined externally with the naked eye and then internally with the aid of a magnifying lens (10×) of a stereomicroscope. Several transverse cuts were made with a clean scalpel for visualization of macroscopic sarcocystosis. The observed macrocysts were physically classified *in situ* based on characteristics such as shape and location.[8,25]

Histopathologic examination. Sixty macroscopic cysts (cystic tissue samples) from 60 infected sheep and goats were dissected along with their surrounding tissues and placed in 10.00% neutral buffered formalin for 48 hr. The tissue samples were dehydrated using different concentrations of alcohol, embedded in liquid paraffin, sectioned at a thickness of 3.00 - 4.00 microtome (µm), and finally were stained with Hematoxylin and Eosin (H & E) for morphological investigation under a light microscope (40×, 100×, and 400×).[26]

Molecular study. For the molecular study, 100 macrosarcocysts samples from different organs of infected sheep (49 esophagi, 23 diaphragms, and 28 abdominal muscles) and 100 macrosarcocysts samples from different organs of infected goats (43 esophagi, 25 diaphragms, and 32 abdominal muscles). The cysts were isolated and washed three times with 0.01 M of phosphate-buffered saline (pH 7.20) at 4.00 ˚C to remove any attached muscle tissues. The obtained cysts were stored in aliquots at –20.00 ˚C before extraction of their genomic DNA.

DNA extraction and polymerase chain reaction (PCR) amplification. For DNA extraction, the frozen cysts (approximately, 20.00-30.00 mg) of each tissue sample from 100 sheep and 100 goats were selected according to the manufacturer's instructions of DNA extraction kit (Jena Bioscience, Jena, Germany). The partial sequences of the extracted DNA were amplified by PCR using two sets of primers, including amplification of D2 region in conserved regions of the *28S rRNA* gene of *Sarcocystis* spp. with the estimated amplicon size of 350 base pair (bp) with primers *SAD2* forward; 5'- GGAAGCCGATTGGAACC -3' and *SAD2* reverse; 5'- CCTTGGTCCGTGTTTCA -3'.[27] The second set of primers, with an amplicon size of approximately 900 bp, was used for the identification of *Sarcocystis* spp. through gene-specific PCR targeting highly conserved *18S rRNA* gene including 2L forward; 5'-GGATAAACCGTGGTA ATTCTATG -3' and 3H reverse; 5'- GGCAAATGCTTTCGCA GTAG -3'.[28] PCR reactions were carried out in 20.00 µL of the reaction mixture containing 10.00 µL of 2X Master mix (Jena Bioscience) containing Taq polymerase, $MgCl_2$, PCR

Table 1. Collection of macrosarcocysts samples from different muscle tissues of naturally infected sheep and goats.

Animals	Inspected animals	Infected animals	Collected macrosarcocysts samples (sample/animal)			
			Esophagus	Abdomen	Diaphragm	Total
Sheep	5,695	955	59	38	33	130
Goats	2,805	382	53	42	35	130
Total	8,500	1,337	112	80	68	260

buffer, and dNTPs, 6.00 µL free ionized water, 1.00 µL (10.00 pmol) of each primer (Macrogean, Seoul, Korea) and 2.00 µL (200 ng) DNA sample. The reactions were performed on a bio-system thermal cycler (Gene-AMP 9700; Applied Bio-systems, Foster City, USA). The thermal profile for the first gene marker (*28S rRNA*) consisted of initial denaturation at 94.00 ˚C for 4 min, followed by 35 cycles of denaturation at 94.00 ˚C for 30 sec, annealing at 54.00 ˚C for 30 sec, extension at 72.00 ˚C for 1 min and final extension at 72.00 ˚C for 7 min.[27] For the second gene marker (*18S rRNA*), the cycling conditions of PCR were as follows: initial denaturation at 95.00 ˚C for 2 min, followed by 40 cycles of denaturation at 94.00 ˚C for 40 sec, annealing at 55.00 ˚C for 35 sec, extension at 72.00 ˚C for 1 min and final extension at 72.00 ˚C for 6 min.[28] Eventually, 8.00 µL of the amplified PCR products were stained by adding 1.00 µL of 11X SYBER DNA stain, analyzed on 1.00% agarose gel and then visualized under UV transilluminator (Gemini BV, Jena, Germany). Good quality of an extracted genomic DNA sample of macroscopic sarcocyst (*S. gigantea*) from sheep (accession No. ON533737) was used as a positive control, while no DNA reaction mixture was used as a negative control for this study.

Sequencing and phylogenetic analysis. A total of 15 PCR products (five esophagi, five diaphragms, and five abdominal muscles) from eight sheep and seven goats were purified by applying a column-based purification kit and sequenced by Sanger sequencing method through an automated sequencer of Macrogen Korean-Company. Species identification was performed through uni-directional sequencing of the amplicons by using the same reverse primers which were used in PCR analysis. All of the newly obtained sequences targeting *18S rRNA* and *28S rRNA* variable genes were submitted and checked by the basic local alignment tool (BLAST) software to obtain the genotypes of the samples. The partial sequences of the target genes from sheep and goats' amplicons were placed in the national center for biotechnology information (NCBI) website under specific accession numbers to be compared with other available *Sarcocystis* DNA sequences in the GenBank® database across the world (Table 2). The phylogenetic analysis was conducted based on nucleotide sequences of the *28S rRNA* and *18S rRNA* target genes and was compared to *Toxoplasma gondii* as an out-group species. The phylogenetic trees were performed using molecular evolutionary genetics analysis (MEGA-X; version 10.1.8) program (http://www.megasoftware.net) to perform the neighbor-joining (N-J) method aligned by CLUSTAL W multiple sequence alignment algorithm, and constructed on the evolutionary distance calculated by the maximum composite likelihood model with 1,000 bootstrap replication as phylogeny test.[29] The reported sequences of sheep and goats in this study were individually placed into two phylogenetic trees.

Statistical analysis. The data obtained from the investigation of the parasitic infection in slaughtered animals were analyzed by applying SPSS Software (version 19.0; IBM Corp., Armonk, USA) using *t*-test and chi-square test. A $p < 0.05$ was regarded as statistically significant.

Results

The total infection rate of macrosarcocysts was 16.77% and 13.62% in sheep and goats, respectively (Table 1). Two types of macroscopic sarcocysts were commonly observed in the muscle tissues of sheep and goats. Fat cysts (average size: 7.50 mm in length and 4.60 mm in width, n = 150) were frequently found in the esophagus and diaphragm, while thin cysts (average size: 3.90 mm in length and 1.95 mm in width, n = 150) were commonly observed in the diaphragm and abdominal muscles. Thin cysts, which were small, rice-white in color and thread-like in shape, were found to be superficially located. Soft creamy fat cysts were seen as bulged and/or embedded in the muscle tissues of the infected animals (Fig. 1).

Table 2. The identified *Sarcocystis* spp. targeting the *18S rRNA* and *28S rRNA* genes compared with other *Sarcocystis* DNA sequences available in the GenBank® database.

Animals	Tissue samples	Species detected	Accession numbers	Partial genes	Identity (%)
Sheep	Esophagus	*S. gigantea*	ON533766	*28S rRNA*	100
Sheep	Diaphragm	*S. gigantea*	ON533737	*28S rRNA*	100
Sheep	Abdominal muscle	*S. medusiformis*	ON533601	*28S rRNA*	100
Sheep	Abdominal muscle	*S. medusiformis*	ON548923	*28S rRNA*	100
Sheep	Esophagus	*S. gigantea*	ON564602	*18S rRNA*	100
Sheep	Diaphragm	*S. medusiformis*	ON548424	*18S rRNA*	100
Sheep	Abdominal muscle	*S. medusiformis*	ON548179	*18S rRNA*	100
Sheep	Abdominal muscle	*S. moulei*	ON544011	*18S rRNA*	99.48
Goat	Esophagus	*S. gigantea*	ON533883	*28S rRNA*	100
Goat	Diaphragm	*S. gigantea*	ON533889	*28S rRNA*	100
Goat	Abdominal muscle	*S. moulei*	ON548922	*28S rRNA*	100
Goat	Esophagus	*S. gigantea*	ON564594	*18S rRNA*	100
Goat	Esophagus	*S. gigantea*	ON596941	*18S rRNA*	100
Goat	Diaphragm	*S. gigantea*	ON548140	*18S rRNA*	100
Goat	Diaphragm	*S. moulei*	ON564624	*18S rRNA*	100

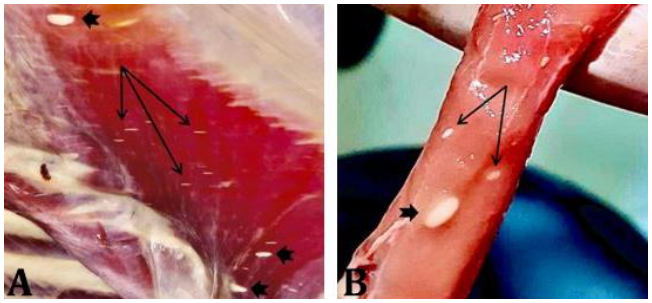

Fig. 1. Macroscopic sarcocysts present in muscle tissues of slaughtered sheep and goats. **A)** Fat cysts (arrowheads) and thin cysts (arrows) in abdominal muscle tissue. **B)** Bulged type of fat cyst (arrowhead) and embedded type of thin cysts (arrows) of macrosarcocysts in the esophageal wall.

Histopathological results of macroscopic sarcocysts in the esophagus, diaphragm and abdominal muscles showed cysts of different shapes and sizes situated either in or above the muscle layers of the tissues. In general, macroscopic sarcocysts consisted of a thick eosinophilic wall with a granular layer immediately underneath, from which septa arise, dividing the cysts into compartments enclosing numerous bradyzoites. However, some cysts were found to have no septa (Fig. 2).

The PCR analysis of all macrosarcocysts samples showed positive diagnostic bands at 350 bp and 900 bp on gel electrophoresis indicating the presence of *Sarcocystis* spp. No band was observed for the negative control (Fig. 3). Sequencing results of the amplified products revealed that the genotypes of fat cysts in the esophagus and diaphragm of sheep and goats were 100% identical to *S. gigantea*, while the genotypes of thin cysts in the diaphragm and abdominal muscles of sheep were 100% identical to *S. medusiformis* and 99.48% identical to *S. moulei*. The genotypes of thin cysts in goats were 100% similar to *S. moulei* compared to other *Sarcocystis* spp. available in the GenBank® database.

Sequencing analysis of *Sarcocystis* spp. indicated that, *S. gigantea*, *S. medusiformis* and *S. moulei* were found in 8 (53.33%), 4 (26.66%) and 3 (20.00%) of the examined animals, respectively. The frequency of *S. gigantea* in sheep and goats was higher than that of *S. medusiformis* and *S. moulei*. The phylogenetic analysis of the *18S rRNA* sequences revealed that *S. gigantea* and *S. medusiformis* isolates were in the same clusters with isolates from other parts of the world. *S. moulei* isolate of goats in our study was similar to the isolate of sheep from Iran (accession No. KP053891.1), and also isolate of sheep in this study was similar to the isolate of goats from Iran (accession No. KC508513.1), (Fig. 4).

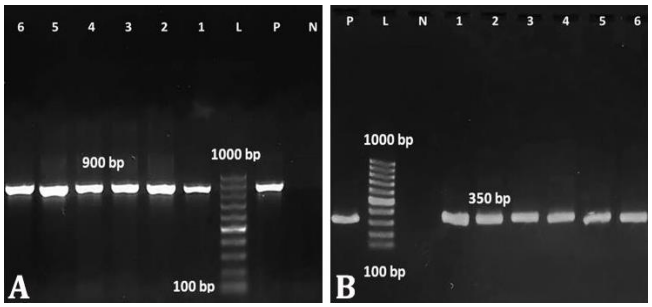

Fig. 3. PCR products of *Sarcocystis* spp. isolated from macrosarcocysts of sheep and goats analyzed on gel electrophoresis. **A)** Partial *18S rRNA* gene of *Sarcocystis* spp. presenting bands at 900 bp. **B)** Partial *28S rRNA* gene of *Sarcocystis* spp. presenting bands at 350 bp. Lane P: positive control, Lane N: negative control, Lanes 1-3: positive samples of the esophagus, diaphragm and abdominal muscle of sheep, Lanes 4-6: positive samples of the esophagus, diaphragm and abdominal muscle of goats, Lane L: 100 bp DNA marker.

The phylogenetic analysis of the *28S rRNA* sequences showed that *S. gigantea* isolates in both of the intermediate hosts in the present study were identical to isolates from Australia (accession No. U85706.1), Egypt (accession No. MT706045.1) and Spain (accession No. MK420025.1). In addition, *S. medusiformis* isolates were clustered with isolates from Egypt (accession No. MT706454.1) and Spain (accession No. MK420026.1), while *S. moulei* isolate of goats in this study matched with an isolate of goats from Australia (accession No. AF012884.1), (Fig. 5).

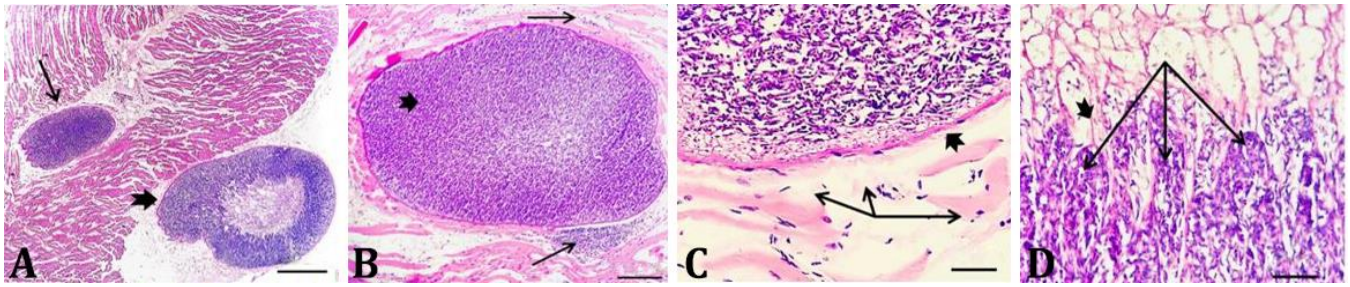

Fig. 2. Histopathological cross-sections of macrosarcocysts isolated from different muscle tissues of slaughtered sheep and goats. **A)** Macroscopic cysts situated in (arrow), and above (arrowhead) the muscle layer of tissue (bar = 500 µm). **B)** A non-compartmented macrocyst contained thousands bradyzoites (arrowhead) surrounded by numerous inflammatory cells (arrows), (bar = 200 µm). **C)** Macrocyst's wall (arrowhead) surrounded by mild edema, mild muscle degeneration and basophilic bradyzoites (arrows) (bar = 20.00 µm). **D)** Thick septa (arrowhead) enclosing the cyst's compartments (arrows) contained numerous basophilic bradyzoites (bar = 20.00 µm).

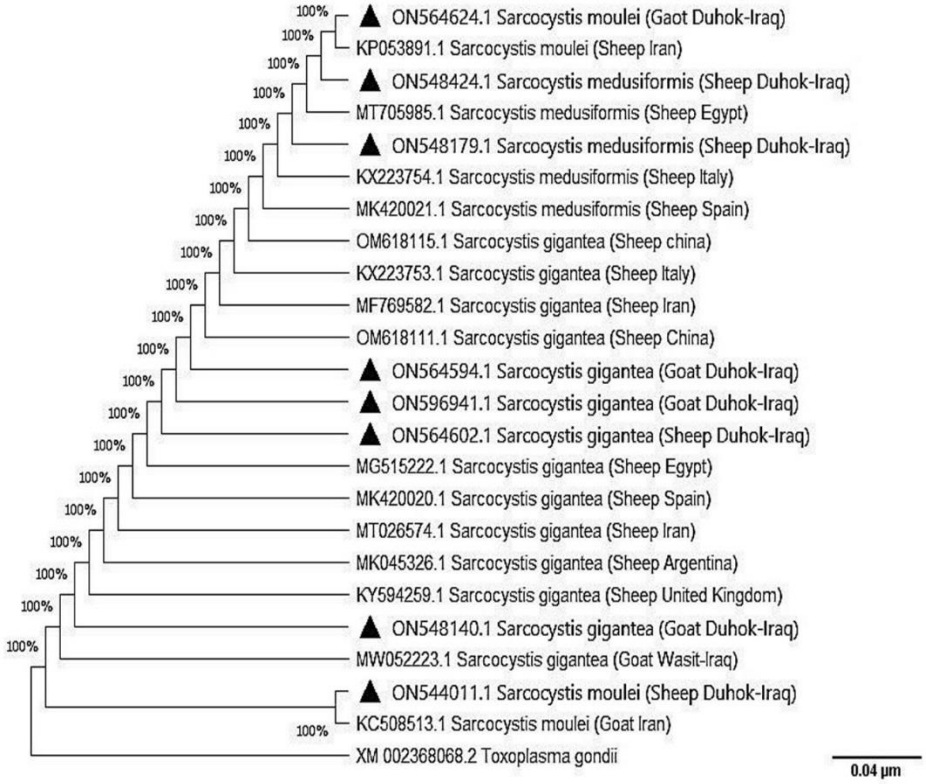

Fig. 4. Phylogenetic tree based on the partial *18S rRNA* sequences of selected *Sarcocystis* spp. performed by the N-J method within the MEGA-X program. The evolutionary distances were computed by the maximum composite likelihood model. All the GenBank® accession numbers corresponded to *Sarcocystis* spp. isolates were followed by their country of origin. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test was 1,000 replicates.

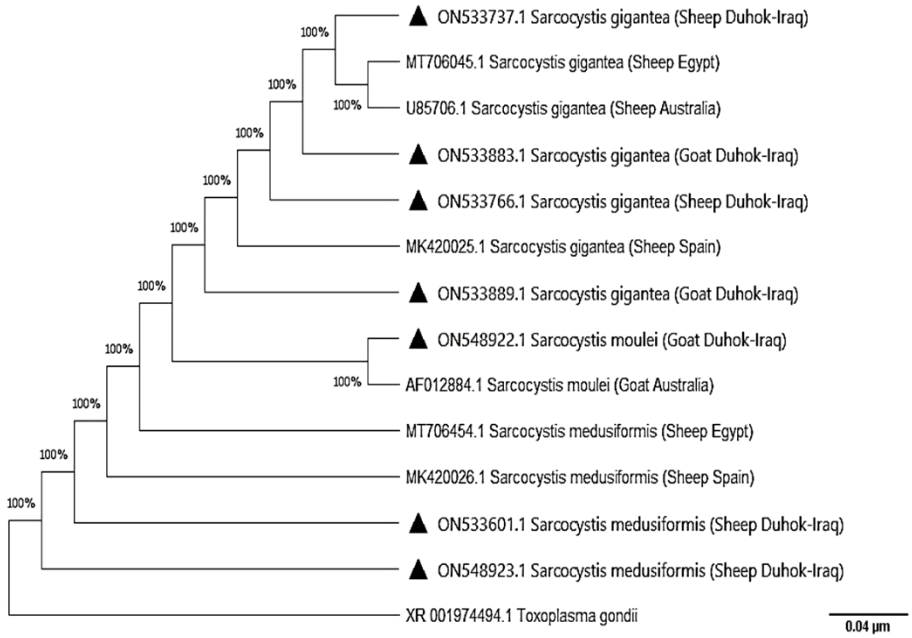

Fig. 5. Phylogenetic tree based on the partial *28S rRNA* sequences of selected *Sarcocystis* spp. implemented by the N-J method within the MEGA-X program. All the GenBank® accession numbers corresponded to *Sarcocystis* spp. isolates were followed by their country of origin. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test was 1,000 replicates.

Discussion

Sarcocystis spp. forming macroscopic sarcocysts are generally considered as non-pathogenic but can cause economic losses in the sheep and goats' meat industries. The presence of these sarcocysts can lead to partial or complete rejection of carcasses of slaughtered ruminants in slaughterhouses, which can negatively affect meat quality and marketing.[17,25] Similar to this study, fat cysts have commonly been found in the esophagus, while thin cysts have been recorded in the diaphragm and abdominal muscles of sheep and goats.[25,30] The overall infection rate of macrosarcocysts in sheep and goats in this study was 16.77%. In other studies, conducted in Iraq, Iran, and Türkiye, the infection rates of macrosarcocysts in slaughtered sheep and goats ranged from 4.10 to 66.00%.[9-15] However, in some studies, no macroscopic sarcocyst was recorded in sheep and goats.[7,31] The differences in the prevalence of *Sarcocystis* infection in these studies could be related to variability in the ingested doses of sporocysts shed by cats (final hosts), as well as differences in the age and immune status of the hosts.[3,5]

In the present study, edema, slight muscle degeneration, necrosis, and approximately mild focal infiltration of mononuclear inflammatory cells were histologically observed surrounding the intact (non-compartmented) and ruptured (compartmented) types of sarcocysts. In two similar studies, macroscopic sarcocysts were histologically found to consist of septa or compartments containing thousands bradyzoites, thick basophilic walls, and mild inflammatory cells infiltrating around the cysts.[32,33] However, in this study, some of the macroscopic sarcocysts were observed to have no septa and were situated deep in the muscle layers of tissues. Only in two studies conducted by Fayer *et al*. in USA. and Decker Franco *et al*. in Argentina, the non-septated type of the genus *Sarcocystis* with no identification of its species has been mentioned.[2,34]

Molecular analysis of the sequenced nucleotides indicated that fat cysts belonged to *S. gigantea*, while thin cysts belonged to *S. medusiformis* and *S. moulei* in sheep and goats, respectively. Similar to our findings, *S. gigantea* isolates from fat cysts were detected in the esophagus, and *S. medusiformis* isolates from thin cysts were found in the diaphragm and abdominal muscles of sheep.[16,35] In contrast to our study, *S. gigantea* isolates from fat cysts were detected in the diaphragm, and *S. medusiformis* isolates from thin cysts were found in the esophagus.[25] Additionally, in a study by Pestechian *et al*. the number of fat cysts belonging to *S. gigantea* was found to be more predominant in the diaphragm than in the esophagus.[36] The molecular detection rate of *S. gigantea* was more frequent than the rate of *S. medusiformis* and *S. moulei* in sheep and goats in this study. In a similar study in Iran, the molecular detection rate of *S. gigantea and S. moulei* isolates from DNA sequence samples of sheep aged from 3 to 7 years old were 66.70 and 20.00%, respectively.[17]

The phylogenetic and alignment results of our isolates targeting *18S rRNA* and 2*8S rRNA* genes showed a high level of similarities and close matching with other *Sarcocystis* species from different geographical areas worldwide. In the present study, cross-infection of *S. gigantea* and *S. moulei* was observed in both sheep and goats. This finding was consistent with results reported in Iran and Saudi Arabia, where sheep were considered as alternative hosts for *Sarcocystis* spp. that originally belong to goats.[17,37] However, this finding was in contrast with other reports that did not find *Sarcocystis* spp. of sheep in goats.[31,38,39] Moreover, Elmishmishy *et al.* in Egypt and Metwally *et al*. in Saudi Arabia reported a strong phylogenetic correlation and cross-transmission of *Sarcocystis* spp. including *S. gigantea* and *S. moulei* in sheep and goats.[40,41] Furthermore, *S. gigantea, S. medusiformis* and *S. moulei* were genetically regarded as sibling sequences as they share about 98.00 - 100% nucleotide homology.[17,21] In a similar study in Iran, *S. medusiformis* was isolated from thin cysts of sarcocyst and considered as a sheep-specific species.[25] *S. gigantea* and *S. moueli* are known as worldwide distributed species, while *S. medusiformis* has only been recorded in some countries such as Iran, Japan, Italy, Jordan, New Zealand, Spain and Australia.[3]

In conclusion, molecular studies on *Sarcocystis* spp. in sheep and goats in Iraq have not been widely performed in Iraq. Therefore, this study aimed to identify *Sarcocystis* spp. forming macroscopic cysts in different muscle tissues of slaughtered sheep and goats using PCR analysis targeting the *18S rRNA* and *28S rRNA* genes, as well as sequencing and phylogenetic analysis. Both *S. gigantea* and *S. moulei* were identified in both sheep and goats, while *S. medusiformis* was only found in sheep. Infection with these species can lead to significant economic losses. Therefore, it is crucial to reduce the contamination of food and water distributed to livestock by keeping cats away from their living areas. Furthermore, preventive measures such as breaking the life cycle of the parasite by avoiding the definitive hosts from consuming the discarded visceral organs of the infected intermediate hosts can help control the infection. Whole sequence analysis of genes encoding *18S rRNA*, and *28S rRNA* or the sequence analysis of other genetic loci is essential to determine the genetic diversity among these *Sarcocystis* spp. Other gene markers, such as *mitochondrial cox1* and *ITS-1*, are also useful in differentiating closely related species of *Sarcocystis*.

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Conflict of interest

The authors declare that there is no conflict of interest.

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