Molecular Identification of *Sarcocystis* Species in Sheep (*Ovis aries*) and Goats (*Capra hircus*) of Duhok Province, Iraq

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**ABSTRACT**

*Sarcocystis* is the most common intracellular cyst-forming protozoan that infects the cardiac, smooth and striated muscles of animals and humans. This parasite has a significant economic, veterinary and medical impact in many regions of the world. The current study aimed to identify *Sarcocystis* species in domestic sheep and domestic goats slaughtered at the slaughterhouses of Duhok province in Iraq, from May 2021 to June 2022 by using molecular study. A total of 250 tissue samples collected from 147 sheep and 103 goats' different organs (79 esophagi, 105 skeletal muscles and 66 hearts) were examined for detection of sarcocysts through microscopic examination of fresh muscle tissues. Consequently, the isolated zoitocysts of sarcocysts and/or their fragments from each infected host were further used for molecular identification. Overall, 98.32% of the examined sheep and 98.06% of the examined goats were microscopically found to be positive for *Sarcocystis* species. Molecular identification of *Sarcocystis* spp. isolates based on analysis of the 18S rRNA gene sequences revealed the existence of *S. tenella* in both sheep and goats and *S. capracanis* in goats only. Alignment and phylogenetic analysis of the isolates in this study showed a very close relationship and close matching with other *Sarcocystis* DNA sequences which were available in the GenBank database. Results of the phylogenetic analysis showed sequences of *S. tenella* as a monophyletic cluster to the sequences of *S. capracanis*. As the 18S rRNA sequences of *S. tenella* and *S. capracanis* showed high similarity with each other, further molecular studies based on the cox1 and ITS1 regions are required for the exact characterization of species identified in the present study. Due to cross-infection and high incidence of *Sarcocystis* spp. in both sheep and goats in the world and in Iraq, development of disease control and prevention measures would be essential.


**INTRODUCTION**

*Sarcocystis* species (Apicomplexa; Sarcocystidae) are intracellular cyst-forming coccidian and zoonotic parasites that infect a wide variety of livestock and humans, with about 200 available species. Most species of the genus *Sarcocystis* have an obligatory two host life cycle; herbivorous, omnivorous or carnivores, in which muscular sarcocystosis develops as intermediate host and omnivorous or carnivorous in which, intestinal sarcocystosis occurs as definitive host (Dubey et al., 2016).

There are four common *Sarcocystis* spp. infecting domestic sheep, *Sarcocystis tenella* and *S. arietcanis* are known as pathogenic and microscopic cyst-forming which transmitted by canids as well as, *S. gigantea* and *S. medusiformis* are known as non-pathogenic and macroscopic cyst-forming that transmitted by felids. Furthermore, goats usually become infected with three different species of *Sarcocystis*, including *S. capracanis* and *S. hercicanis* regarded as pathogenic and microscopic cyst-forming for which canines act as definitive hosts, and *S. moulei* considered as non-pathogenic and macroscopic cyst-forming for which felines act as definitive hosts.
spp. are more specific to their intermediate hosts than to their final hosts. In addition, Sarcozystis spp. that are transmitted by canines are found to be more prevalent and more pathogenic than those species that are transmitted by felines (Lindsay and Duby, 2020).

The prevalence of cyst forming Sarcozystis spp. in domestic and wild sheep and goats in different countries across the world were ranged from 45% to 100% (Dubey et al., 2016; Anvari et al., 2020). In Iraq, sarcocystosis is an endemic disease with infection rate reached 90-100% (Latif et al., 1999; Zangana and Hussein, 2017). In Iran, the prevalence ranged from 36.83% to 100% (Mirzaei and Rezaei, 2016; Hooshyar et al., 2017; Pestehchian et al., 2021). In Egypt, it was 40-100% (Elmishmishy et al., 2018). And in Turkey, the prevalence was 55-100% (Beyazit et al., 2007). Depending on the morphological characteristics using light microscopy for detection of Sarcozystis spp. in Iraq, Barham et al. (2005), reported the infection of goats with S. capracanis and S. moulci in Baghdad, and Abdullah (2011), reported the infection of sheep with S. tenella and S. arieticanis in Sulaymaniah.

The morphological characters of sarcocysts based on observation of wall types of the cysts such as thick or thin and palisade-like villar protrusions or hair-like villar protrusions by light and transmission electron microscopy (TEM) are usually used for identification of Sarcozystis spp. by many authors (Dubey et al., 2016). Although, morphological methods are beneficial for detection of parasitic infection, they are not precisely reliable to distinguish the specific or closely related Sarcozystis species. Therefore, several molecular diagnostic techniques such as Polymerase Chain Reaction (PCR) and its variants have been developed for specific identification of Sarcozystis spp. using different molecular markers, such as 18S rRNA and 28S rRNA gene regions as well as internal transcribed spacer 1 (ITS1) and mitochondrial cytochrome c oxidase subunit 1 (cox1) sequences (El-Morsey et al., 2019; Gjerde et al., 2020). Although the mitochondrial cox1 sequence is considered as a more precise molecular marker for Sarcocystisidae, the 18S rRNA gene region has extensively been used to distinguish apicomplexan as well as the genus Sarcozystis from other eukaryotic species due to its hyper-variable regions interspersing within highly-conserved DNA sequences (Fischer and Odenberg, 1999; Stoeckel et al., 2012). Furthermore, the databases comprise more 18S rRNA sequences of the genus Sarcozystis which are ready to be used for species identification than other molecular gene sequences (El-Morsey et al., 2019; Imre et al., 2019). These characters make the 18S rRNA gene a valuable diagnostic marker.

Meanwhile, no molecular study was conducted for identification of Sarcozystis spp. in Iraq. Hence, the present study was conducted to identify Sarcozystis spp. in domestic sheep and goats using PCR, sequencing and phylogenetic analysis based on the 18S rRNA gene.

MATERIALS AND METHODS

Sample collection: Two hundred fifty tissue samples were randomly collected from 147 slaughtered sheep (46 esophagi, 37 hearts, and 64 skeletal muscles) and 103 slaughtered goats (33 esophagi, 29 hearts, and 41 skeletal muscles) aged from 1 to 2 years in the slaughterhouses (Sumail, Zakho, and Amedia) of Duhok province, Kurdistan region of Iraq, from May 2021 to June 2022. Each heart, skeletal muscle (25-30 g) and whole esophagus (70-100 g) sample was individually placed in a self-sealing plastic bag, transported in an ice box to Parasitology Research Center at College of Veterinary Medicine, University of Duhok and then stored at 4°C for further investigations.

Macroscopic examination: The macroscopic examination was performed immediately after collection of the tissue samples. Several cross-cuts were made using a clean scalpel blade in the heart and skeletal muscle for observation of macroscopic sarcocysts. The whole tissue of the esophagus was longitudinally segmented in order to expose its lumen; additionally, its external and internal walls were macroscopically examined for the existence of macrosarcocysts (Bittencourt et al., 2016).

Microscopic analysis of fresh tissues: The esophagus, heart and skeletal muscle tissues were microscopically examined for detection of microscopic sarcocysts using squash and grinding methods. In squash preparation, the tissue samples (2-3 mm) were compressed between two glass slides and observed under a light microscope at 10x magnification power (Latif et al., 2015). In tissue grinding method, approximately 15-20 g of each tested sample was minced thoroughly in a blender, mixed with 30 ml of phosphate buffered saline (pH: 7.2), and then stirred on a magnetic stirrer with a magnetic bead for 10 minutes until a complete homogenization. Moreover, the mixture was filtered by a strainer mesh, collected in a clean test tube and then centrifuged at 600 xg for at least 10 minutes. Moreover, the supernatant was removed, and the sediment was re-suspended with 15 ml of PBS buffer. Then, the mixture was transferred into a clean petri-dish for visualization and measurement of sarcocysts under an optical microscope at 10x and 40x magnifications (Imre et al., 2019). Eventually, several complete and/or fragments of the cysts (5-10 cysts/ sample) of each positive sample were put in 1.5 ml DNAse and RNAse free microtubes containing 80-100 µl of free-ionized water, and then stored at -20°C for molecular analysis.

DNA extraction and PCR amplification: The genomic DNA of the collected sarcocysts was extracted through a commercial DNA extraction Kit (Jena-Bioscience, Germany) according to the manufacturer's instructions with a modification in the final addition of buffer by adding 10 µl to the extracted DNA instead of 100 µl (Bittencourt et al., 2016). PCR analysis was carried out for 100 positive samples (34 esophagi, 36 skeletal muscles and 30 hearts) of collected microcysts from 50 sheep and 50 goats. A 900 base pair (bp) fragment of the 18S rRNA gene of Sarcozystis spp. was amplified by PCR using the primer pair; 2L (5'- GGATAAACCGTGGTAATTCT ATG -3') as forward and 3H (5'- GGCAAATGCTTTC GCAGTAG -3') as reverse (Imre et al., 2019). PCR reaction was performed in 20µl of the reaction mixture containing 10 µl of 2x Master Mix (Taqpolymerase, PCR buffer, MgCl2, and dNTPs), 1µl (10pmol) of each primer,
2µl (200ng) of DNA sample, and 6µl of free-ionized water. The reaction was performed in a thermocycler (ABI, Gene-Amp, 9700 PCR, 96-well thermal cycler). The cycling conditions applied consisted of initial denaturation for 2 minutes (min) at 95°C, followed by 35 cycles of denaturation for 45 seconds (s) at 94°C, annealing for 30 s at 55 °C, extension for 1 min at 72°C, and final extension for 7 min at 72°C. Finally, 1 µl of 1× SYBER DNA stain (Jena Bioscience, Germany) was added into 9 µl of each amplified PCR product to be analyzed on 1% agarose gel and visualized under UV transilluminator. A good quality of genomic DNA extracted from sarcocysts (accession no. ON564602) of sheep from a previous study was used as a control positive, and ultra-pure water was used as a negative control in this study.

**Sequencing and phylogenetic analysis:** A total of six positive amplicons (2 esophagi, 2 skeletal muscles and 2 hearts) of the infected sheep and goats were purified using a column-based purification kit (Thermo-scien; USA), and then sequenced by Sanger sequencing method through an automated sequencer (Perkin-Elmer ABI; USA, Macrogen LTD Korea). Sequencing of the nucleotides was performed by using 10 µl of the same reverse primer which was used for the PCR reaction. The obtained nucleotide sequences were searched and matched with other published Sarcocystis DNA sequences in the GenBank database by the BLAST search tool on the NCBI website to obtain Sarcocystis spp. genotypes from the samples. Furthermore, the partial sequences of the 18S rRNA regions of Sarcocystis isolates were submitted to the NCBI submission portal under accession numbers; PO363930, PO363951, OP363953, OP363955, OP363963, and OP376570. Moreover, the sequences of Sarcocystis isolates and other related GenBank-retrieved Sarcocystis sequences across the world were deposited and analyzed using MEGAX software to determine their phylogenetic relationships based on the 18S rRNA gene sequences. Multiple sequence alignment was performed by applying muscle alignment method. Furthermore, the sequences of the particular genes were cut at both ends to provide the homologous nucleotide positions. The phylogenetic tree was constructed through applying the neighbor joining (N-J) method based on evolutionary distances computed by the Kimura two-parameter model with the 1000 replicates bootstrap value. *Eimeria tenella* was used as an out-group species.

**RESULTS**

In the present study, no macroscopic sarcocysts were observed in the hearts and skeletal muscles of the 101 slaughtered sheep and 70 slaughtered goats. Furthermore, macroscopic sarcocysts were also not detected on the outer and inner layers of the 79 examined esophagi of the hosts. Out of 250 examined samples, 247 (98.8%) samples were found to be infected with microscopic sarcocysts in sheep and goats similarly through both of the fresh tissue microscopic-examination techniques. Moreover, microscopic sarcocysts were found in 146 (99.32%) out of 147 examined sheep and 101 (98.06%) out of 103 examined goats. The microscopic cysts were predominantly obtained from 67 (100%) samples out of 67 hearts, followed by 77 (98.72%) esophagus tissues (n=78), and 103 (98.10%) samples out of 105 skeletal muscles. In general, the cysts isolated from esophagus, skeletal muscle and heart of sheep and goats were found to be elongated ovals, measuring; 198-595 µm × 50-150 µm (363±107 µm × 86±38 µm, n=30) and slender, measuring; 625-1,188 µm × 30-70 µm (750±235 µm × 50±14.5 µm, n =30) in shape with thick palisade-like protrusions or finger-like protrusions wall, measuring; 2.5-7.4 µm (4.3±1.2 µm, n=30) enclosing numerous banana shaped bradyzoites, measuring; 7.5-15 µm × 2.5-3.5 µm (12.80±1.9 µm × 3.55±0.6 µm n=30) among and/or in striated, non-striated and cardiac muscle fibers of sheep and goats (Fig. 1a, Fig. 1b).

PCR analysis of microscopic sarcocysts from the infected sheep and goats showed a specific band (900 bp) on gel electrophoresis, which was indicating the presence of *Sarcocystis* spp. No band was found in the lane of negative control. The sequencing analysis and alignment results showed that the genotypes of the isolates from sheep and goats in this study were 99% to 100% identical to *S. tenella* and *S. capracanis* which were reported in different countries across the worldwide (Table 1). In the present study, the sequences of *S. tenella* and *S. capracanis* in both sheep and goats shared 99.30–99.95% nucleotide homology with themselves under 84–99% of query covers based on the 18S rDNA gene level.

The phylogenetic relationship's analysis of the isolates in this study showed that, *S. tenella* haplotypes of sheep and goats in the phylogenetic tree placed in the same cluster with haplotypes originated from other countries such as Norway, Egypt, Poland, Iran, Spain, India, China, and Argentina, and Hungary. Similarly, the *S. capracanis* isolate of goats in this study was closely placed with isolates of the same host from China and Iran (Fig. 2). The consequences of the phylogenetic analysis showed sequences of *S. tenella* as a monophyletic cluster to sequences of *S. capracanis* in both of the hosts.

**DISCUSSION**

The macroscopic sarcocysts which are characteristic of *S. medusiformis* and *S. gigantea* in sheep and *S. moulei* in goats were not observed in any of the examined animals. In Iraq, domestic sheep and domestic goats intended for production of meat are commonly reared on grass and in close contact with stray dogs more than with stray cats; this could be one of the main causes for the absence of macrosarcocysts in the examined animals. *Sarcocystis tenella* in sheep and *S. capracanis* in goats are the most predominant pathogenic species inducing microscopic cysts. Macroscopic sarcocysts are visible by the naked eye and can easily be removed by veterinarians in the slaughterhouses, while the microscopic sarcocysts are neglected because they are invisible in nature; hence it remains in the meat, repeat its life cycle and lead to enormous economic losses (Lindsay and Dubey, 2020). Therefore, promoting prevention and control strategies for parasitic infection are essential. Tissue cysts of *S. tenella* and *S. capracanis* are typically found in the esophagus, diaphragm, heart, skeletal muscle, liver, and brain of
Table 1: *Sarcocystis* spp. isolates of sheep and goats targeting the 18S rRNA regions compared with the most related *Sarcocystis* DNA sequences present in the GenBank database.

<table>
<thead>
<tr>
<th>Animals</th>
<th>Organs</th>
<th><em>Sarcocystis</em> spp.</th>
<th>GeneBank Numbers</th>
<th>Highest identities with other isolates</th>
<th>Differences from the closest isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sheep</td>
<td>Esophagus</td>
<td><em>S. tenella</em></td>
<td>OP363953</td>
<td>100%</td>
<td>no difference</td>
</tr>
<tr>
<td>Sheep</td>
<td>Skeletal muscle</td>
<td><em>S. tenella</em></td>
<td>OP363930</td>
<td>100%</td>
<td>no difference</td>
</tr>
<tr>
<td>Sheep</td>
<td>Heart</td>
<td><em>S. tenella</em></td>
<td>OP363951</td>
<td>99.70%</td>
<td>(A) insertion at 641 position</td>
</tr>
<tr>
<td>Goat</td>
<td>Esophagus</td>
<td><em>S. tenella</em></td>
<td>OP363955</td>
<td>100%</td>
<td>no difference</td>
</tr>
<tr>
<td>Goat</td>
<td>Skeletal muscle</td>
<td><em>S. tenella</em></td>
<td>OP376570</td>
<td>100%</td>
<td>no difference</td>
</tr>
<tr>
<td>Goat</td>
<td>Heart</td>
<td><em>S. capracanis</em></td>
<td>OP390169</td>
<td>99.17%</td>
<td>substitutions at 145 (G-A), 161 (G-T) and 170 (C-T) positions</td>
</tr>
</tbody>
</table>

Fig. 1a: Microscopic examination of tissue cysts from sheep and goats. A and B) Elongated oval microcysts in and out of the muscle fibers of skeletal and esophagus after tissue grinding and squash preparation (scale bar = 100 µm). C) A slender shaped microcyst detected by tissue compression of skeletal muscle (scale bar = 100 µm). D) Numerous bradyzoites surrounding the cyst of heart muscle after rapture by tissue grinding method (scale bar = 50 µm).

Fig. 1b: Light microscopic appearance of *Sarcocystis* spp. in wet mounts. A) Fragment of a slender shaped sarcocysts of *S. tenella* in skeletal muscle of sheep with several internal septa forming compartments enclosing bradyzoites (scale bar = 20 µm). B) A portion of *S. tenella* wall with abundant villus or finger-like protrusions (arrow), (scale bar = 10 µm). C) A mature spindle shaped sarcocysts of *S. capracanis* released from heart muscle of goat containing large number of bradyzoites with irregularly shaped wall (scale bar = 50 µm). D) A head of *S. capracanis* wall with densely packed palisade-like protrusions (arrow), (scale bar = 10 µm).

Sheep and goats (Hong et al., 2016). The cysts morphology was ranged from elongated oval to slender in shape with recorded dimensions of 140-1690 µm in length and 30-130 µm in width (Barham et al., 2005; Hu et al., 2016; Marandykina-Prakienė et al., 2022). In addition, bradyzoites of the cysts were found to be banana in shape with measurement size 8-13.5 µm x 1.5-3.5 µm (Kutty et al., 2015; Latif et al., 2015; Hong et al., 2016). The severity of clinical symptoms of sarcocystosis in sheep and goats depends on the ingested dose of sporocysts, which were shed by carnivores, and the immune status of the hosts (Fayer et al., 2015). In acute sarcocystosis, the
Fig. 2: Phylogenetic tree showing relationship of 18S rRNA gene sequences of Sarcocystis spp. identified in the present study with those available in GenBank. The percentage value of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Bootstrap values below 50% are not shown. Names in parenthesis next to the Sarcocystis spp. names and their accession numbers are referred to animal species, organs and countries of the isolates. Black symbols refered to isolates of Sarcocystis spp. identified in the present study.

Sarcocystis spp. are known to cause clinical symptoms such as anorexia, fever, anemia, muscle weakness, abortion, encephalitis, encephalomyelitis and even death. Chronic sarcocystosis can lead to economic losses due to emaciation and a decrease in animal productions such as meat, milk and wool (Fayer et al., 2015; Lindsay and Dubey, 2020). Clinical symptoms such as inflammation, myocarditis and hepatitis were also reported in experimentally inoculated sheep with sporocysts of S. tenella from canine feces (Dubey et al., 2016). The incidence of microsarcocysts in sheep and goats in this study was compatible with results reported in Turkey, Iran, Saudi Arabia, and Egypt ranged from 80%-100% (Beyazit et al., 2007; Dehaghi et al., 2012; Al-Quraishy et al., 2014; Elmishmishy et al., 2018). The high infection rate of the parasite could be related to close contact of hosts with carnivores or might be due to persistent keeping of dogs with sheep and goats’ herds for safety issues, in addition to rapid maturation (within 1 to 2 months) of the cysts in muscle tissues of the intermediate hosts after ingestion of sporocysts (Dubey et al., 2016).

In this study, S. tenella and S. capracanis were identified in sheep and goats through molecular analysis of 18S rRNA gene sequences. Sarcocystis tenella was isolated from the esophagus, skeletal muscle and heart of sheep, in addition to the esophagus and skeletal muscle of goats, and S. capracanis was isolated from heart of goats. The cysts of S. tenella were globally regarded as a specific species for sheep and they have been isolated from the cardiac, smooth, and striated muscles of the hosts by several authors from various countries (Hu et al., 2017). While, in a similar study in Korea, S. tenella was isolated from muscle tissues of goats rather than of sheep (Hong et al., 2016). In a case report from Iran, S. tenella was also isolated from brain of sheep (Selahi et al., 2014). In contrast, S. capracanis was regarded as an alternate parasite for other herbivorous and was reported in the cerebrospinal fluid of two sheep with nervous clinical signs in United Kingdom (Formisano et al., 2013). Moreover, S. capracanis was identified in wild sheep species in Spain and Austria (Delgado-de Las Cuevas et al., 2021; Prakas et al., 2021), and S. tenella was identified in goat-antelope species (Rupicapra rupicapra) in Poland (Kolenda et al., 2015) based on the 18S rRNA and cox1 gene sequences. Nevertheless, the situation of the morphologically similar species pair S. tenella/S. capracanis in regard to their life cycle within the Caprinae intermediate host group appears to be not finally elucidated and further studies are needed to clarify the intermediate host status of the Caprinae species (Prakas et al., 2021). Meanwhile, sheep and goats are regarded as alternate hosts for transmission of Sarcocystis species; S. tenella of sheep and S. capracanis of goats are genetically considered as sister sequences due to their close phylogenic correlation at the 18S rRNA gene sequence level (El-Morsey et al., 2019). Sarcocytis capracanis was
most closely related with *S. tenella* based on 18S rRNA, 28S rRNA, and mitochondrial cox1 sequences, as it shares identities of 95.7-99.1, 95.3 and 92.3-93.2% with other *S. tenella*, respectively (Hu et al. 2016). However, using cox1 as the preferable genetic marker for discrimination of sibling sequences of *Sarcocystis* spp. in sheep and goats could be significant (Gjerde et al., 2020).

**Conclusions:** This is the first molecular study to identify and confirm *S. tenella* and *S. capracanis* in domestic sheep and domestic goats in Iraq. High incidences of infection by *Sarcocystis* species-forming microcysts in muscle tissues of slaughtered sheep and goats intended for human consumption were detected in the slaughterhouses of Duhok province, north of Iraq. The identified species of *Sarcocystis* (*S. tenella* and *S. capracanis*) are the most common pathogenic ones in sheep and goats which cause significant economic losses. Therefore, it’s essential to prevent the contaminated food and water with sporocysts to livestock. In addition, keeping dogs out of livestock would help prevent the contaminated food and water with sporocysts which cause significant economic losses. Therefore, it’s essential to discriminate apicomplexan as well as the genus *Sarcocystis* from other eukaryotic species due to its hyper-variable regions interspersing within highly conserved DNA sequences, applying mitochondrial cox1 as the preferable genetic marker for discrimination of sibling sequences of *Sarcocystis* spp. in sheep and goats could be significant. Furthermore, a comparative study using genetic markers, such as 28S rRNA, cox1 and ITS1 are also required for more precise molecular differentiation of *S. tenella* and *S. capracanis* in both sheep and goats.

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**Authors contribution:** Assistant Professors; Dr. Assel Anwar Ibrahim and Dr. Mohammed Shukri Shukur designed and supervised the current research study. Corresponding author (PhD Student; Shivan Nawzad Hussein) collected the samples and investigated them at the laboratory of Duhok Research Center, College of Veterinary Medicine, University of Duhok. The supervisors have also helped in the analysis of the data and proof writing of the manuscript.

**Conflicts of Interest:** No conflict of interest exists.

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