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Morphological and molecular identification of *Sarcocystis arieticanis* isolated from the cardiac muscles of domestic sheep and goats

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Abstract

Sarcocystis species are the most common intracellular protozoans and zoonotic parasites that infect the cardiac, striated, and non-striated muscles of omnivores, herbivores, carnivores, reptiles, fishes, and humans. This study aimed to detect and identify the existence of *Sarcocystis* species in the cardiac muscles of sheep and goats through a combination of morphological and molecular analysis. The morphological analysis relied on the cyst's wall structures obtained from the cardiac muscles of both hosts showed thin wall cysts with irregularly arranged hair-like protrusions. Furthermore, the molecular study based on the 18S rRNA genes found that the cysts were most closely related to *S. arieticanis* with 99.46% nucleotide homology. The total prevalence of sarcocystosis in sheep and goats via microscopical investigation was 98.70%, while it was 100% via molecular analysis. Sequencing and phylogenetic analysis illustrated that *S. arieticanis* has a strong phylogenetic correlation and regarded as a sibling species with a monophyletic cluster to *S. tenella* of sheep and *S. capracanis* of goats, in which canines are their definitive hosts. Due to the high frequency of sarcocysts infection and their impacts on the host's health status and economy in Iraq and in other countries, hygienic policies and preventative measures are essential for controlling the disease. To the author's knowledge, this is the first molecular and morphological study achieved to identify *S. arieticanis* in sheep and goats in Iraq.

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Introduction

Sarcocystis is an abundant zoonotic and parasitic disease of the Phylum Apicomplexan that forms intracellular cysts in the muscle tissues of domestic and wild animals (1). More than 200 species of *Sarcocystis* have been recognized globally by many authors from several countries (2). The life cycle of these protozoans typically requires two hosts: herbivorous, in which muscular sarcocystosis (asexual stage) form occurs in the intermediate hosts, and carnivorous, in which intestinal sarcocystosis (sexual stage) form occurs in the definitive hosts (3). The genus *Sarcocystis* can form two types of cysts in intermediate hosts: macroscopic sarcocysts that can be seen by the naked eyes, and microscopic sarcocysts which cannot be seen by visual examination (4). Sheep's *Sarcocystis* spp. can either form macrocysts with feline definitive hosts, for instance, *S. gigantea* and *S. medusiformis*, or microcysts with canine definitive hosts, such as *S. arieticanis* and *S. tenella* (5,6). Moreover, goats can develop macroscopic *Sarcocystis* spp. in which felines are their final hosts, such as *S. molei*, or microscopic sarcocysts, such as *S. capracanis* and *S. hercicanis* with canine's definitive hosts (7). Recently, *Sarcocystis* spp. which were considered specific for sheep or goats, were reported in both of the intermediate hosts, i.e., sheep can be infected with goat's *Sarcocystis* spp., and goats can also become infected with sheep's *Sarcocystis* spp. (7-9). For that reason, cross-transmission of *Sarcocystis* spp. between the two hosts can occur (10). Ovine and caprine sarcocyst infections were reported from several countries worldwide and ranged from 10% to 100% (11,12). Different morphological and molecular methods have been developed to detect, identify, and differentiate *Sarcocystis* spp. Light and electron microscopy have been performed to detect the infection based on the cyst's wall structure. In contrast, molecular analysis based on several molecular markers, for instance; 18S rRNA, 28S rRNA, COX-1, and ITS-1 regions have applied for identification and differentiation of *Sarcocystis* spp. in final and intermediate hosts (13-15).

The current study, which relied on a combination of morphological characteristics and molecular analysis, aimed to identify *Sarcocystis* spp. that infect domestic sheep and goats.

Materials and methods

Ethical approve

Ethical approval was obtained from College of Veterinary Medicine, University of Duhok, date: 1.06.2023, issued no. CVM023/0106UoD.

Collection of samples

During the period of this study, which started from December 2022 to August 2023, a total of 154 cardiac muscle samples from 86 slaughtered sheep and 68 slaughtered goats aged from 1 to 2 years were randomly collected from different slaughterhouses (Duhok, Zakho, and Sumail) located in Duhok governorate, Iraq. Each of the collected muscle samples from each slaughtered animal was put in a sealed plastic bag labeled with number, date, and animal species preserved in an ice box and then transferred to the center of research at the Veterinary Medicine College, Duhok University for microscopic and molecular analysis.

Microscopic examination

The fresh cardiac muscle tissues of sheep and goats were examined the same day after collection. Approximately 20 gm of each muscle tissue was homogenized well by an electronic blender and then added into 25 - 30 ml of phosphate-buffered saline. Then, the mixture was put in a clean glass flask, placed on a magnetic stirrer for complete homogenization, and sifted through a fine strainer mesh in a glass petri dish for detection and morphological investigation of *Sarcocystis* spp under a light microscope at 10 X and 40 X magnification powers. Eventually, the observed complete sarcocysts and/ or fragments of the cysts were isolated through a 1 ml pipette and then put in DNase and RNase-free microtubes to be stored under - 20 ℃ for molecular study (16).

Molecular analysis

The genomic DNA of the isolated sarcocysts and/ or fragments of the cysts were extracted from 20 sheep and 20 goats' cardiac muscles according to the manufacturer's instructions of a commercial DNA extraction kit (Jena Bioscience, Germany). The extracted DNA of these protozoans was preserved in a freezer under - 20 ºC for further analysis. PCR amplification based on the 18S rRNA gene region (~ 900 bp) for sarcocysts DNA samples conducted through using 2 sets of primers: 2L forward (5' - GGATAAACCGTGGTAATTCTATG - 3') and 3H reverse (5' - GGCAAATGCTTTCGCAGTAG - 3') as described by Imre *et al*. (16). The amplification reaction was implemented in a 20 µl reaction mixture holding 10 µl of PCR 2 × Master Mix (Jena Bioscience, Germany), 1 µl (10-pmol) of each forward and reverse primer (Macrogean, Korean-Company), 2 µl of DNA sample-template (150 - 200 ng/ µl), and 6 µl of nuclease-free water. The thermal cycling conditions started at 94 ˚C (2 min) for initial denaturation, followed by 40 cycles of denaturation at 95 ˚C (40 s), 54 ˚C (35 s) for annealing, 72 ˚C (1 min) for extension, and 72 ˚C (6 min) for the final extension. To validate the result before sequencing, the amplified PCR products of each infected animal were stained with safe dye, electrophoresed in 1.5% agarose gel, and then investigated under a UV transillumінator. This study used a good quality of PCR product (~ 200 ng/ µl) of sarcocyst spp. (Accession No. OP363951) that obtained from the cardiac muscle of an infected domestic sheep from a previous study as control positive. In addition, the PCR nuclease-free water was used as control negative.

The PCR amplicons from each infected sheep and goat were purified by a PCR clean-up kit (Macrogen, Seoul-Korea) and sent to the macrogean company in South Korea for sequencing by applying the Sanger sequencing method based on the same primers which were used in the PCR reaction. The obtained sequences of sarcocysts samples after neucleotide sequencing were submitted to GenBank database to identify their genotyes. Similarities between the obtained sequences of the isolated species were compared with other *Sarcocystis* spp. published in the GenBank database by applying the NCBI/ BLAST program. Phylogeny of the isolated species of *Sarcocystis* was investigated in the MEGA-X software, and a tree was constructed based on the 18S rRNA region inferred by the maximum parsimony (MP) algorithm applying the subtree pruning regrafting (SPR) method. *Eimeria tenella* was used as an out-group spp.

Statistical analysis

The collected data was analyzed using SPSS software (IBM version 18.0, USA). The t-test and chi-square (χ2) test were used to find the variations in the infection rate obtained from sheep and goats. A P value of > 0.05 was considered a significant value.

Results

Sarcocysts were microscopically found in the cardiac muscles of 85 (98.84%) sheep out of 86 samples and 67 (98.53%) goats out of 68 samples. Morphologically, the sarcocysts in both of the intermediate hosts found to be microscopic and ribbon-shaped, ranging from 462 - 1474 × 71 - 115 µm (880 ± 300 × 75 ± 13.5 µm, n = 35). The walls of the cysts found covered with thousands of thin-delicate, irregularly arranged hair-like protrusions measuring 3.2 - 6.3 µm (3.7 ± 0.9 µm, n = 35). The sarcocysts were found to be compartmented by several septa and packed with a large number of crescent-shaped bradyzoites, ranging from 9.0 - 14.0 µm × 3.4 - 5.4 µm (11.8 ± 1.2 × 3.5 ± 0.4 µm, n = 35) as in figure 1. Statistically, no differences were found to be significant in the frequency rate of sarococystosis in both of the hosts.

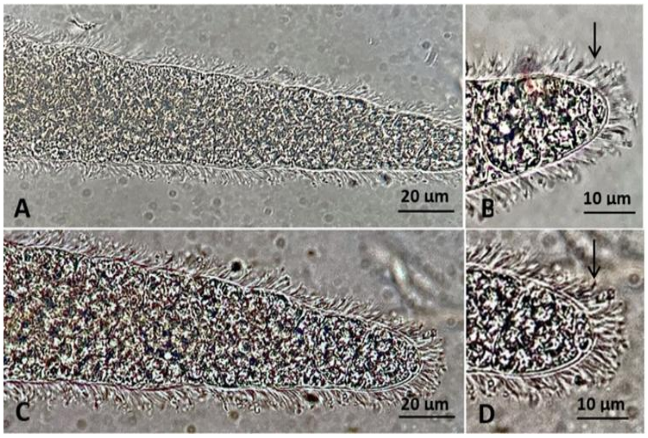

Figure 1: Wet mount appearance of *Sarcocystis* spp. isolated from the cardiac muscles of sheep and goats under a light microscope. A) Ribbon-shaped sarcocyst obtained from the cardiac muscle of sheep (scale bar = 20 µm). B) A part of the sarcocyst's wall covered by irregular hair-like or villus (arrow) protrusions (scale bar = 10 µm). C) A mature sarcocyst isolated from the cardiac muscle of goats with fragments containing numerous cystizoites (scale bar = 20 µm). D) A part of the sarcocysts' wall surrounded by numerous hair-like (arrow) or villus protrusions (scale bar = 10 µm).

The DNA was extracted from all cardiac muscle samples of the examined animals. The 18S rRNA regions of sarcocysts DNA templates were successfully amplified through PCR analysis. Gel electrophoresis investigation of the isolates revealed a specific DNA fragment size (~ 900 bp), indicating the presence of *Sarcocystis* spp. (Figure 2).

After nucleotide sequencing, two comparable sequences of the 18S rRNA regions were isolated from two different sarcocysts DNA isolates of sheep and goats. The two 18S rRNA nucleotide sequences, each obtained from distinct microsarcocysts, were 854 bp in size and were found to be approximately identical. Therefore, only one nucleotide sequence (OP781963) of isolate (I26) from sheep was submitted to the GenBank software. In addition, sequence similarities searches of the obtained sequence with other *Sarcocystis* spp. in the GeneBank using NCBI/ BLAST program showed 99.49% identity with those of *S. arieticanis* from other countries worldwide. The phylogenetic tree based on the partial 18S rRNA gene deposited the newly obtained sequence of *S. areticanis* (OP781963) within a similar cluster, including the sequences of *S. arieticanis* (MT729807 and MT729808) from Iran, *S. tenella* (MK420018) and *S. capracanis* (MW832494 and MW832492) from Spain. These species were found to form a group using canines as final hosts (Figure 3). The phylogenetic study found *S. arieticanis* to be a sibling species to *S. capracanis* and *S. tenella* from sheep and goats, respectively.

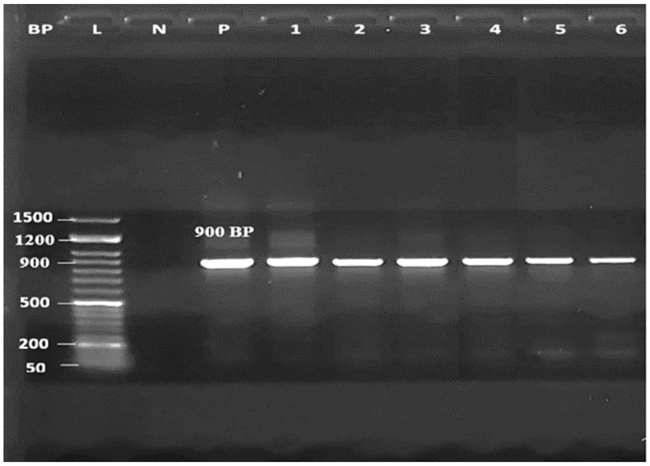

Figure 2: Evaluation of *Sarcocystis* spp. through PCR analysis targeting the 18S rRNA regions on 1.5 % agarose gel electrophoresis that presented bands at approximately 900 bp. Lane BP: Base pare, lane N: Negative control, lane P: Positive control, lanes 1 – 3: Positive samples from heart muscles of sheep, lanes 4 – 6: Positive samples from heart muscles of goats, and lane L: 50 bp DNA marker.

Figure 3: Phylogenetic tree of *Sarcocystis* spp. constructed on the 18S rRNA sequences and inferred by maximum parsimony (MP) algorithm using subtree pruning regrafting (SPR) method. GenBank accession numbers are placed in front of the taxon names of *Sarcocystis*. The values that appear among the branches represent the bootstrap value percent per 1000 replicates. The bootstrap values under 50% are not presented. The new sequence of *S. arieticanis* (OP781963) has the taxon name with a triangle mark.

Discussion

The most common microscopic and pathogenic *Sarcocystis* spp. are thought to be *S. tenella* and *S. arieticanis* in sheep, as well as *S. capracanis* and *S. hercicanis* in goats (17,18). The canines are the definitive hosts of these species, which can cause acute symptoms in livestock animals such as fever, anemia, emaciation, abortion, myositis, placentitis, endocarditis, encephalitis, and even death (2,5,19). Microscopical analysis revealed that these cysts had thick walls with protrusions resembling fingers. Based on the morphological features, *S. tenella* and *S. capracanis* were frequently reported from the heart, diaphragm, brain, esophagus, and skeletal muscles of sheep and goats (11,15,20). Additionally, through using light and electron microscopy, it was found that the sarcocysts of *S. areiticanis* and S. *hercicanis* had a greater affinity for the cardiac muscles and were morphologically distinguished by small, hair-like protrusions that were unevenly organized on the cyst's surface (21-23). In addition, based on the morphological characteristics by wet mount analysis, these cysts were also found in the mouth and diaphragm of sheep and goats (24,25).

In this study, cysts of *S. arieticanis* were microscopically found in the cardiac tissue of both intermediate hosts depending on the characteristics of the cyst's wall. Similarly, *S. arieticanis* cysts were shown as ribbon-shaped, measuring 137 - 1693 × 75 - 130 µm. The walls of the cysts were thin and had frequent unevenly arranged hair-like or villus protrusions, measuring up to 4.0 - 7.5 µm in length. *S. arieticanis* cysts were found to be septate, and their internal sections were fully packed with banana-shaped cystizoites, measuring 9.5 - 14.0 × 2.5 - 5.0 µm (26,27). Although microscopical analyses are appropriate for detecting morphological features of sarcocysts infection, they need not be applied as confirmatory tests. In order to distinguish between the closely related *Sarcocystis* species, molecular characterizations with sequencing and phylogenetic analysis are essential.

In this study, the molecular percentage rate of *Sarcocystis* infection in goats and sheep was 100%. Researchers from various countries have also reported similar molecular infection rates of *Sarcocystis* spp. reaching 99-100% in sheep and goats, respectively (28-30). In Iraq, Al-Diwaniyah province, the molecular percentage rate of sarcocysts infection in sheep was 97% (31). In the Wasit province, it was 89% of small ruminant populations (32). Based on various gene markers, such as 18S rRNA, 28S rRNA, ITS-1, and COX-1 sequences, molecular analysis of morphologically thick-walled sarcocysts with finger-like protrusions were identified as *S. tenalla* and *S. capracanis* in sheep and goats, respectively. Moreover, *S. arieticanis* in sheep and S. *hercicanis* in goats are cysts with morphologically thin walls and delicate hair-like protrusions (15,25,33). However, in the muscle tissues of Iraqi domestic goats (*Capra hircus*), Hussein *et al*. (10) observed thick-walled finger-like protrusion cysts that were molecularly identified *as S. tenella.* In addition, a study by Delgado-de las Cuvas *et al*. (34) molecularly identified *S. capracanis* from thick-walled *Sarcocystis* spp. in wild sheep species (Barbary sheep) from Spain. *S. tenella* was identified in a Polish goat-antelope species (*Rupicapra rupicapra tatrica*) by Kolenda *et al*. (35). Furthermore, it showed that the *S. capracanis* which regarded to form microscopic types of cysts in the intermediate hosts of goats were found in the European mouflons (*Ovis gmelini musimon*) (26).

In this study, the thin-walled hair-like protrusion cysts isolated from the heart muscles of sheep and goat hosts molecularly belonged to *S. arieticanis* based on the 18S rRNA gene level. Although sheep considered as a specific for *S. arieticanis* species, the species was also detected in goat's intermediate hosts. This finding was compatible with studies that regarded goats as alternative hosts for species naturally found in sheep (8,10,36). Furthermore, it has been suggested that sheep and goats, which have similar dietary preferences, lifestyles, and feeding practices, could transmit *Sarcosystis* spp., particularly those of *S. arieticanis, S. hercicanis*, *S. tenella*, and *S. capracanis* (7,37).

The alignment and phylogenetic analysis placed the obtained sequence of *S. areiticanis* in the same cluster as *Sarcocystis* spp., in which canines are definitive hosts, especially of *S. arieticanis*, *S. capracanis*, and *S. tenella*. This finding was supported by many authors from different countries (11). Based on distinct gene markers with 93-97% nucleotide homology, investigations by Hussein *et al*. (23), El-Morsey *et al*. (25), and Metwally *et al*. (38) revealed that *S. arieticanis* is a species that is very closely linked to *S. hercicanis*, and *S. tenella* is a species that most closely related to *S. capracanis*. Furthermore, *S. arieticanis* and *S. tenella* from Lithuanian sheep shared 97.54 - 97.93% nucleotide homology, according to research by Marandykina-Prakienė *et al*. (30). While in a study by Hu *et al*. (33) showed that, *S. hercicanis* isolates (KU820984 and KUB20985) from goats were closely belonged to *S. arieticansis* isolate (L24382) with 97.3 - 97.4% nucleotide similarities and *S. tenella* isolates (KC209734 and KP263754) with 95.3- 95.6% homology from sheep, followed by *S. capracanis* from goats (L76472) with 95.3 - 95.4% identities by molecular analysis based on the 18S rRNA gene regions. In sheep, *S. tenella*, *S. arieticanis*, *S. hercicanis*, and *S. capracanis* in goats were phylogenetically regarded as sibling sequences and monophyletic clusters (38). Meanwhile, cross-transmission of the mentioned species can be found in both of the intermediate hosts; improvement of the preventative measures for controlling these parasites, such as breaking the life cycle of the parasite by preventing the final hosts from consuming raw and infected meat of the intermediate hosts is essential (39).

Conclusion

This is the first morphological and molecular study that confirms the presence of *S. areiticanis* in domestic sheep and goats in Iraq. *S. areiticanis* is regarded as the most prevalent pathogenic species, causing veterinary and economic impacts due to the persistent keeping of the dog's final hosts with sheep and goat's intermediate hosts for protection issues. The sarcocysts in sheep and goats were morphologically found to have hair-like protrusions on the wall's surface. Furthermore, depending on PCR amplification of the 18S rRNA gene and sequencing of the sarcocyts DNA samples, the cysts were recorded as *S. areiticanis*. Phylogenetic relationships and alignment results showed that *S. areiticanis* was very closely associated with *S. areiticanis*, *S. tenella*, and *S. caparacins* of sheep and goats from other countries. Therefore, cross-infection with these species may occur in sheep and goats.

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Conflict of interest

There is no conflict of interest.

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التعرف الشكلي و الجزيئي للحويصلات الصنوبرية المعزولة من العضلات القلب في الاغنام و الماعز

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الخلاصة

ان الانواع العائدة لطفيلي الحويصلات الصنوبرية تصيب العضلات القلبية، والعضلات المخططة وغير المخططة في اكلات اللحوم والاعشاب والزواحف والاسماك و كذلك الانسان. هدفت الدراسة الحالية، التعرف على انواع المتكيسة العضلية التي تصيب عضلات القلب في الاغنام والماعز بالطرائق الشكلية والجزيئية. شكليا اعتمد الفحص على شكل وتركيب جدار الكيس الموجودة في عضلة القلب في كلا المضيفين من حيث سمك جدار الكيس وترتيب البروزات الشعيرية عليها. وجدت الدراسة الجزيئية التي شيدت على جين 18S rRNA بأن الاكياس المعزولة ترتبط ارتباطا وثيقا بطفيلي الحويصلات الصنوبرية مع تماثل النيوكليوتيدات بنسبة ٩٩,٤٦٪. كما اظهرت النتائج ان نسبة الاصابة بطفيلي المتكيسة العضلية مجهريا بلغت ٩٨,٧٠٪ و جزيئيا بلغت ١٠٠٪. وعند تحليل شجرة النشوء الجيني لوحظ وجود تطابق جيني بين الانوع *S. tenella*, *S. arieticanis* و *S. capracanis* في الاغنام و الماعز. نظرا لانتشار العالي لطفيلي الحويصلات الصنوبرية في العراق وبقية الدول وكذلك لتسببها خسائرالاقتصادية العالية فضلا عن الخسائر الصحية، فإن تطوير قواعد النظافة والتدابير الوقائية أمر ضروري للسيطرة على المرض. تعد هذه الدراسة الأولى من نوعها في العراق التي اجريت جزيئيا على هذا النوع من طفيلي المتكيسة العضلية.