

## Research Article

# Molecular and Microscopic Investigation of *Sarcocystis* Species Isolated from Sheep Muscles in Iran

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*Sarcocystis* species is a genus of cyst-forming parasites infecting both humans and animals globally. Some of these species cause clinical and subclinical diseases in the host and may lead to economic losses. This study was carried out to identify the distribution patterns of *Sarcocystis* spp. in slaughtered sheep based on the digestion method and PCR-RFLP in Isfahan, the center of Iran. In total, 150 fresh muscle samples (30 hearts, 60 esophagi, and 60 diaphragms) were investigated by naked eye observation and then scrutinized based on the digestion method. To this end, pepsin and HCl were used to observe the *Sarcocystis* parasite via a light microscope. The PCR was carried out to intensify a fragment of the 18S rRNA gene. Afterward, the PCR products were exposed to digestion by endonuclease *TaqI*, *HindII*, *EcoRI*, and *AvaI*. Consequently, the results of RFLP were confirmed by sequencing, and the phylogenetic placement of all species was analyzed. Through the examination by the naked eye, 5/150 (3.33%) macroscopic cysts were found in the samples. With the tissue digestion and microscopic examination, 116 (77.33%) samples were positive for *Sarcocystis* spp.; however, 125 (83.33%) samples were positive with PCR. Moreover, the results of sequence analysis on macrocysts and microcysts showed that 4% and 96% of the species belonged to *S. gigantea* and *S. tenella*, respectively. According to the results of the current study, sarcocystosis caused by *S. tenella* are highly prevalent among sheep in the Isfahan region. Due to the high prevalence of *Sarcocystis* infection in the world and Iran, the development of disease control and prevention policies in sheep would be essential, and changing attitudes in the way of keeping livestock from the traditional type to the industrial method is recommended in this regard.

## 1. Introduction

*Sarcocystis* species is among the most prevalent cyst-forming protozoan parasites with worldwide distribution in various hosts [1, 2]. Its life cycle circulates among carnivores and omnivores as definitive hosts and intermediate hosts, including herbivores, omnivores, and carnivores [3, 4]. More than 200 species of *Sarcocystis* with different clinical symptoms were reported all over the world [5]. Livestock animals, such as sheep, are among the most infected hosts

with global distribution [6]. Various species of *Sarcocystis* are identified in sheep, such as *Sarcocystis gigantea* and *S. medusiformis*, which are nonpathogenic and cause macroscopic cysts; moreover, the cats are definitive hosts for them. In contrast, some species, such as *S. tenella* and *S. arieticanis* should be considered pathogenic causing microscopic cysts, and canids are the definitive host for them [7, 8]. Mature sarcocysts of various *Sarcocystis* spp. can be differentiated by distinct phenotypic features, such as form, size, cyst wall thickness, and organization of villar

protrusions. However, these features may be affected by the location and stages of the cyst development, age of the parasite, kind of host cell, and other conditions of the parasitized cell. Therefore, molecular techniques are a reliable way to confirm species identification and differentiation [9, 10]. Based on previous studies, a valuable target utilizes the variable regions of the 18S rRNA gene to identify and characterize diverse species even from the identical genus [11, 12]. The symptom of sarcocystosis in various hosts is different and depends on the parasite species and the host immune system status [3, 13]. Infection with pathogenic *Sarcocystis* spp. can cause morbidity and mortality. Moreover, the other potential symptoms might be weight loss, anorexia, fever, anemia, abortion in the sheep, and other intermediate hosts with considerable economic losses [2, 14]. There are different reports of infection rates in sheep from 36.83% to 100% in various parts of Iran [15–18]. Therefore, species identification can be an essential issue due to the high prevalence of the parasite, especially in Iran, and different clinical signs in various species [19–21].

The current study aimed to characterize *Sarcocystis* spp. which was isolated from sheep using tissue digestion and 18S rRNA gene restriction fragment length polymorphism (PCR-RFLP) on the tissue samples of sheep in Isfahan, in the center of Iran.

## 2. Materials and Methods

**2.1. Sample Collection.** In total, 150 fresh muscle samples (30 hearts, 60 esophagi, and 60 diaphragms) were taken from 150 slaughtered sheep in Isfahan Province, the center of Iran. Subsequently, the tissue samples obtained from each organ were examined by the naked eye for macrocyst. To this end, serial cuts of the muscles and digestive methods were performed with the aim of detection and confirmation of *Sarcocystis* bradyzoites [21, 22].

**2.2. Microscopic Examination of the Fresh Tissues.** Muscle digestion was carried out in pepsin-hydrochloric acid according to the other authors' instructions [21]. Briefly, 20 grams of pooled muscles from different tissues of the same animal were incubated for 20 min at 40°C in 50 ml of acid-pepsin solution (pepsin 2.6 g (Merk, Germany), NaCl 5 g (Merk, Germany), and 1 M HCl 7 ml (Spain, Scharlab S. L.) plus 993 ml of distilled water). Afterward, the digested samples were strained through a 38 mm sieve, centrifuged at 2000 g for 5 min, and the sediments were suspended in 0.5 ml of distilled water [21, 23]. A drop of the suspension was examined to observe the presence of *Sarcocystis* bradyzoites by using the light microscope at 400X magnification.

**2.3. Molecular Study.** Phosphate-buffered saline (PBS) was used to wash the sediments of digested infected muscles three times, and the zoites were collected in small conical tubes and kept frozen at -20°C [21]. At this stage, the genomic DNA was extracted from positive samples based on the phenol-chloroform purification technique as previously

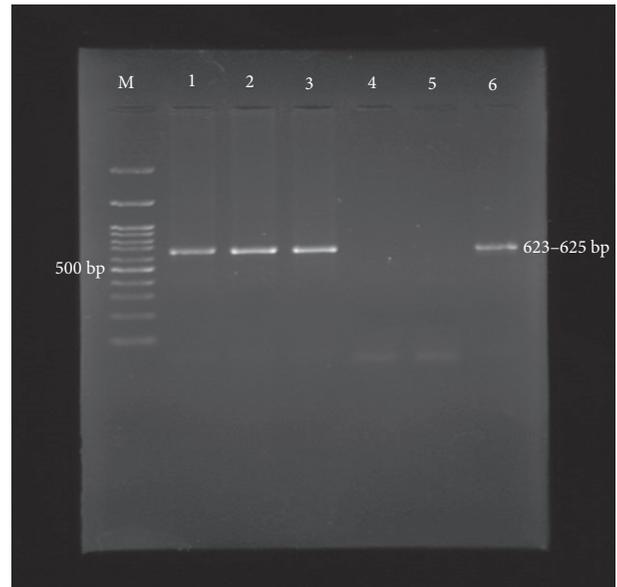


FIGURE 1: Gel electrophoresis of 18S ribosomal DNA (623–625 bp) among studied sheep tissues with *Sarcocystis* spp. M, 100 bp DNA marker; 1, 2, and 3, samples; 4, negative control of *Neospora caninum*; 5, negative control of *Toxoplasma gondii*; and 6, positive control.

described [24]. The extracted DNA was preserved at -20°C for further studies. A 623–625 bp fragment of the 18S ribosomal DNA gene was amplified by PCR reaction after applying the following primers: forward (SarcoF: '5'-GCACTTGATGAATTCTGGCA - 3') and reverse (SarcoR: '5'- CACCACCCATAGAATCAAG - 3') [13]. Reactions took place on a gradient thermal cycler (BIO-RAD T100 Thermal Cycler, Singapore). The PCR amplification was performed with an initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C (2 min), 57°C (30 s), and 72°C (2 min), and a final extension step at 72°C (5 min). To evaluate the results, the PCR product was stained with safe stain electrophoresed in a 1% agarose gel and visualized on a UV transilluminator. As a negative control, the DNA of *Neospora caninum* and *Toxoplasma gondii* was also analyzed by the mentioned primers simultaneously to establish the probability of the cross reaction with the mentioned protozoans. The PCR products of macrocysts and microcysts were then subjected to RFLP using *Ava*I, *Hind*II, *Taq*I, and *Eco*RI restriction enzymes. The reaction was carried out with 10U of each restriction enzyme, 1X specific buffer, and 10 µl PCR products. Subsequently, they were incubated for 16 h at 37°C according to the manufacturer's recommendation. The digestions were analyzed using agarose gel electrophoresis alongside the 50 bp DNA ladder. Afterward, the digested PCR products were run on a 3% agarose gel and envisaged with safe stains under ultraviolet light [13]. For the confirmation of RFLP results, four PCR products of macrocysts and six PCR products of microcysts were sequenced and checked by the BLAST tool on the NCBI website. Phylogeny of all species was analyzed with MegaX software, and a tree was constructed using the Maximum Composite Likelihood algorithm.

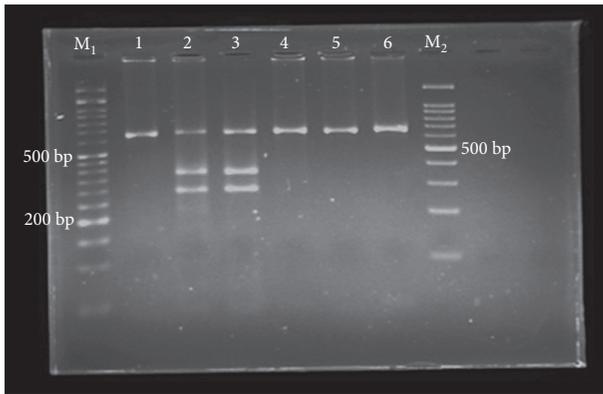


FIGURE 2: RFLP gel electrophoresis of *Sarcocystis* spp and 18S ribosomal DNA after digestion with different endonucleases. M<sub>1</sub>, 50 bp DNA marker; 1, positive control; 2 and 3, TaqI; 4, AvaI; 5, EcoRI; 6, HindII; and M<sub>2</sub>, 100 bp DNA marker.

### 3. Results

Out of 150 samples, 125 (83.33%) samples were infected with *Sarcocystis*. Only five macroscopic *Sarcocystis* were found in the samples. Furthermore, the microscopic cysts were found in 116 (77.33%) out of 150 examined sheep using the tissue digestion method. The most predominant microscopic cysts were obtained from 53 (88.33%) diaphragm samples ( $n = 60$ ), followed by 43 (71.66%) samples out of 60 esophagus tissues and 20 (66.66%) samples out of 30 hearts. Four (80%) samples out of 5 macrocysts were found in the diaphragm, and one (20%) of them was in the esophagus. The PCR reaction using the mentioned primers confirmed an expected band on the agarose gel, which is a section comprising 623–625 consensus nucleotides (Figure 1). The results of the molecular method revealed that 83.33% of the samples had *Sarcocystis* spp., and the highest rate of infection was detected in diaphragm samples (93.33%), followed by 83.33% and 73.33% in the heart and esophagus tissues, respectively. *TaqI* enzyme produced restriction fragments of 344 bp and *EcoRI* 10 bp, as well as bands without any digestion with *AvaI* and *Hind II* representing *S. tenella* (Figure 2). The sequencing results indicated that all samples of microsarcocysts and macrosarcocysts belonged to *S. tenella* and *S. gigantea*, respectively. Furthermore, there were not any cross reactions with *T. gondii* and *N. caninum* in the PCR results. The phylogenetic analysis of the 18S rRNA sequence from the various geographical regions revealed that all *S. gigantea* isolates were in the same cluster with isolates from Spain (accession no. MK420020.1), Norway (accession no. KC209733.1), and Egypt (accession no. MG515222.1); moreover, all *S. tenella* isolates clustered with isolates from Iraq (accession no. LC364049.1) and Iran (accession no. MT569891.1) (Figure 3).

### 4. Discussion

In this study, heart, diaphragm, and esophagus tissues were implemented to investigate the presence of *Sarcocystis* spp. According to previous studies, these organs are the most

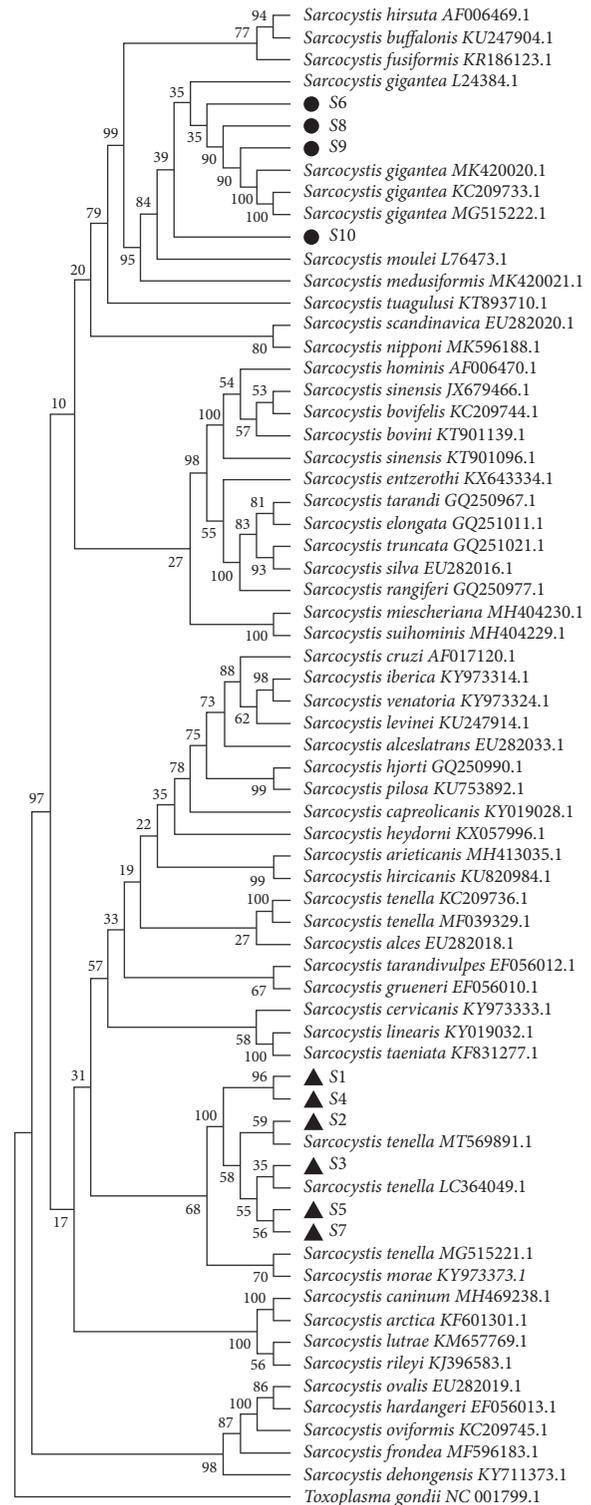


FIGURE 3: Evolutionary history was concluded using the UPGMA method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method. Evolutionary analyses were conducted in MEGA X.

common sites for *Sarcocystis* infection in the sheep [9, 16–19, 25, 26]. The results revealed the high

predominance of *Sarcocystis* infection among sheep slaughtered in the Isfahan slaughterhouse. This rate of infection in sheep in this area indicates a high exposure rate of these animals to the sporocysts, which are shed with dogs, cats, and possibly wild animals that can be served as the definitive hosts. Previous studies have reported four species of *Sarcocystis* from sheep [7, 27]; however, according to the recently conducted studies, up to six species have been identified from sheep [4]. The result of our study indicated that the isolates related to *S. tenella* and *S. gigantea* and the lack of identification of other species may be due to the small sample sizes. The parasite may lead to clinical symptoms, such as reduced weight, abortion in cattle, and finally, economic loss; in addition, it may lead to nausea, stomachache, and diarrhea after eating undercooked or raw meat in humans. Among these species, *S. tenella* and *S. arieticanis* are pathogenic and contribute to clinical symptoms, such as abortion, fever, anemia, and anorexia in sheep [28]. In contrast, *S. gigantea* and *S. medusiformis* are nonpathogenic, while they may cause the low quality of meat and economic losses [7]. In the present study, only five macrosarcocysts were detected. It might be as a result of the definitive host derivation of microsarcocysts since the interaction between sheep and dog in the section is high or it may be related to the low number of samples. According to a study by Latif et al., the great quantities of macroscopic and microscopic *Sarcocystis* were 4.10% and 97% among Iraqi sheep under observation, respectively [29]. Several factors, such as host specificity, the morphology of cyst, and ultrastructure of the cyst wall, in addition to molecular and biochemical features, were used for the identification of *Sarcocystis* spp. [21]. For detection and differentiation among *Sarcocystis* in meat samples on slaughtered carcasses, various common genus-specific methods have been used, including staining with methylene blue, doh-smear, digestion, and histology [30]. Some serological methods, including enzyme-linked immunosorbent assay and an indirect fluorescent antibody test, have been utilized for the detection of sarcocystosis in sheep and some other animals as well. Nonetheless, their limitations make it difficult to use these techniques regularly. One of the critical limitations is the considerable antigenic similarities and consequent cross reactions with other *Sarcocystis* spp. The serological methods cannot be used for species identification along with genus determination [31]. Over recent years, molecular methods have been implemented with high sensitivity and specificity for the species classification of *Sarcocystis* spp. secluded from diverse samples [11, 21]. Based on several studies, the variable regions of the highly conserved 18S ribosomal subunit are useful genetic markers for differentiating the species of *Sarcocystis* in different hosts, such as sheep [11, 32]; however, according to recently performed studies, *cox1* is better for *Sarcocystis* species identification in sheep [4]. Based on the results of the current limited study, *S. tenella* and *S. gigantea* present among the studied sheep in Isfahan province, and infections with other *Sarcocystis* spp. were not detected. Moreover, there were not any cross reactions with *T. gondii*

and *N. caninum* in the PCR results. In the current study, molecular investigation demonstrated that all detected *Sarcocystis* belonged to *S. tenella* and *S. gigantea*. In previous similar studies throughout the world, *Sarcocystis tenella* was reported with different prevalence rates in Mongolia (96.9%), Ethiopia (93%), Turkey (47.3%–86.5%), Iran (33.9%), and the United States (84%) [6]. Consistent with the results of a study conducted by Oryan et al. and Heckerroth, as well as Tenter, *Sarcocystis* is mainly observed in the esophagus, larynx, and lingua muscles [25, 33]. The present study revealed the highest rate of infection in the diaphragm. By the same token, in Lorestan and Khuzestan provinces, Hamidinejat et al. reported that all *Sarcocystis* isolated from slaughtered sheep belonged to *S. gigantea* [13]. On the other hand, the detection of *S. gigantea* in this region of Iran probably demonstrated that the differential examination was not applied when acute clinical sarcocystosis related to *S. tenella* and *S. arieticanis* happened; moreover, sheep disorders, such as abortion, were attributed to other protozoa [13, 27]. Since morphological characters are various in different conditions, such as location and developmental stage of the cysts, the reliable method to confirm morphological species identification is molecular techniques. In this study, phylogenetic analysis showed that six *S. tenella* haplotypes in the phylogenetic tree were placed closed to a haplotype of *S. tenella* originated from different countries, such as Egypt, Iraq, and China. Similarly, isolated *S. gigantea* species in this study were closely located near the species from Spain and Norway. These may be the result of low nucleotide and haplotype variation of *Sarcocystis* spp. and global transmission of the parasite through animal transference.

## 5. Conclusions

The results of this study provided data about the frequency of sarcocystosis that was more prevalent among sheep in Isfahan, the center of Iran. Most of the identified species are *S. tenella*, which are pathogenic in sheep; accordingly, it is necessary to take health measures to prevent high economic losses.

## Data Availability

Data are available upon request to the corresponding author.

## Conflicts of Interest

The authors declare that they have no conflicts of interest.

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