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Original Article

Molecular Survey on *Sarcocystis* Species in Slaughtered Sheep in Hamedan, Iran

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ABSTRACT

Introduction: *Sarcocystis* is an apicomplexan heteroxenous protozoan leading to adverse consequences for production in sheep with remarkable importance in public health. The current study aimed to investigate molecular prevalence data on *Sarcocystis* spp. in slaughtered sheep using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method in Hamedan, Iran.

Methods and materials: The carcass of 60 sheep slaughtered in the Hamedan abattoir were sampled from May 2019 to June 2020. Heart, scapular, esophageal and diaphragmatic muscles were macroscopically examined and recorded as positive in case of the presence of tissue cyst. In this regard, 50 grams of each muscle was sliced and transferred to the laboratory on ice for microscopic and molecular analysis. The molecular identification of *Sarcocystis* spp. was performed using the PCR-RFLP method.

Results: By microscopy, all specimens contained bradyzoites in cysts. The molecular analysis further revealed *Sarcocystis* species, including *Sarcocystis gigantea* (*S. gigantea*), *S. tenella,* and *S. arieticanis*.

Conclusion: The present study emphasized that three *Sarcocystis* spp. were circulating among sheep and carnivorous hosts in the investigated area. Further molecular investigations are highly recommended to better evaluate the epidemiology of this zoonotic parasite.

1. Introduction

Sarcocystosis is a global parasitic disease of the musculoskeletal system in various animals, such as mammals, birds, reptiles as well as humans[1]. *Sarcocystis* spp. is an obligatory intracellular heteroxenous parasite, circulating among definitive carnivorous hosts (canids and felids) as predators and herbivorous intermediate hosts, such as cattle, sheep, goat, buffalo, and camel as prey[1]. Sexual reproduction occurs in definitive hosts, shedding sporocysts into the environment versus feces. In contrast, asexual reproduction and schizogony occur in striated and heart muscles of intermediate hosts, leading to Sarcocystis formation[1]. Hence, ingesting food/water contaminated with sporocysts and devouring carcasses infected with sarcocyst are the main routes of transmission in intermediate and definitive hosts, respectively[2]. In this sense, consumption of highly infected meat from food animals is not suitable for humans[2].

The most widespread *Sarcocystis* species include *Sarcocystis tenella* (*S. tenella*), *S. arieticanis*, *S. medusiformis,* and *S. gigantea* (*ovifelis*) in sheep as well as *S. hominis* (*bovihominis*), *S. cruzi* (*bovicanis*) and *S. hirsute* (*bovifelis*) in cattle. *Sarcocystis* infections in the definitive hosts are frequently asymptomatic, while they may inflict mild disease by *S. gigantea* in sheep, to acute disease by *S. cruzi* in cattle and *S. capracanis* in goat[3]. Cellular degeneration, hemorrhage, and inflammatory lesions are the most observed pathologies induced by the parasite[4]. Invasion of the nervous system is rare, except for *S. neurona*, which develops the nervous disease in some animal species[4]. In pregnant sheep, the acute disease may result in abortion, fetal death, and still birth[5]. The most prominent pathologic traits of the infection develop following merozoite release in the circulation[6]. Generally, the parasite undergoes two merogony cycles. The first-generation meronts are formed 1-2 weeks upon infection in endothelial cells of blood

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vessels in most body organs, and the second-generation meronts emerge 3-5 weeks post-infection, leading to systemic intravascular coagulation, perivascular inflammation, necrosis, and hemorrhage[7]. Fever directly correlates to the rate of parasitemia, resulting in anemia due to the constant bleeding related to extravascular hemolysis on an immunologic basis. Animal death occurs after ingestion of a huge number of sporocysts, which usually coincides with second-stage merogony[7]. *Sarcocystis* species involving canid hosts may result in fetal death and abortion in infected, non-immune animals. Nevertheless, no exact underlying mechanism is determined for *Sarcocystis*-induced abortion, and the parasite has been rarely isolated from the infected fetus and placenta under experimental conditions[8].

Diagnosis of the infection in livestock depends on several approaches, including abattoir-based macroscopic examination of infected organs in animal carcasses, microscopic observation of tissue cysts using digestion and/or stamp method as well as trichinoscopy, along with immunohistochemical and molecular techniques[9]. Bradyzoites of various *Sarcocystis* spp. released by the digestion method could not be differentiated due to the morphological similarities in the bradyzoite's shape[9]. Several serological-based methods are available for detecting sarcocystosis in sheep such as enzyme-linked immunosorbent assay (ELISA) and an indirect fluorescent antibody test (IFAT), with significant sensitivity in the late phase of infection[10]. However, discrimination between sheep pathogenic and non-pathogenic *Sarcocystis* spp. is impossible using such methods[10]. With the advent of molecular techniques, species identification of parasitic agents has been facilitated, which provides a deeper understanding of *Sarcocystis* epidemiology[11].

In Iran, the prevalence rates of *Sarcocystis* infection among sheep and cattle populations were reported at 80% and 100%, respectively. With this in mind, the present cross-sectional study aimed to investigate *Sarcocystis* prevalence rate and involved species in sheep of Hamedan, western Iran, using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method.

2. Materials and Methods

2.1. Muscle sampling and microscopic examination

A total of 60 samples were collected. Upon referral to an abattoir, Hamedan, Iran, from May 2019 to June 2020, a total number of 20 (10 male and 10 females with the age range of 1-3 years) sheep infected with macroscopic cysts were sampled, and 50 grams of each muscle of heart, scapular, esophageal, and diaphragmatic muscles were necropsied. A total number of 40 sheep lacking macroscopic cysts were also randomly sampled in a similar manner and all specimens were tagged and transferred on ice to the laboratory of the Veterinary Medicine Department of Sanandaj Islamic Azad University, Sanandaj, Iran.

2.2. Digestion method

For microscopic examination, the digestion method was used[12]. Briefly, a digestion solution (containing 5.2 g pepsin, 10 ml hydrochloric acid, and 100 ml buffered phosphate, Merck Germany) was applied to each muscle specimen.

Figure 1. Macrosarcocysts of *Sarcocystis gigantean* in striated muscle of infected sheep

Next, the yielded digesta was passed through a clean piece of textile and then centrifuged (Memmert, Germany) at 1500 rpm for 10 minutes. After supernatant elimination, smears were prepared from the remaining pellet, dried, and fixed with methanol. Finally, smears were stained with diluted Giemsa solution (20-30 minutes) and observed under a light microscope (Olympus, Japan) using 100x magnification. Observation of the bradyzoite stages of the parasite was reported as a positive sample (Figure 2).

2.3. DNA extraction and PCR-RFLP analysis

For the purpose of DNA extraction, muscle samples kept at -20°C were initially put at the ambient temperature. Next, the procedure of DNA purification was done using the Cinnagen tissue DNA extraction kit (Cinnagen Company, Iran), according to the manufacturer's instructions. The DNA was pelleted using ethanol and stored at -80°C for further use[13]. In the following Sar-F1 (5 GCA CTT GAT GAA TTC TGG CA 3) and Sar-F2 (5' CAC CAC CCA TAG AAT CAA G3) primer pairs were used for the amplification of the target gene, in the context of a thermal cycle as 94°C for 5 minutes (initial denaturation), 30 cycles of 94°C for 2 minutes (denaturation), 57°C for 30 seconds (annealing), 72°C for 2 minutes (extension), along with a final extension step at 72°C for 5 minutes. The final PCR products were

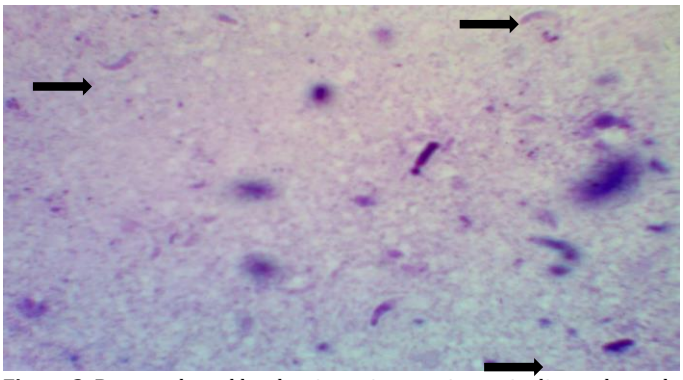

Figure 2. Banana-shaped bradyzoites microscopic cyst in digested muscle tissues

kept at refrigerator temperature (-20°C)[13]. The PCR reaction (total volume = 30 µl) was prepared by mixing 12 µl distilled water (DW), 3 µl X10 buffer, 2 µl 1.75 mM $MgCl_2$, 0.5 µl of each primer (10 pmol), 10 µl template DNA, and 0.5 µl Taq polymerase enzyme. After amplification, 5 µl of PCR product was electrophoresed on 1% agarose gel containing DNA-safe stain, and likely bands were visualized using a GelDoc apparatus[14].

The *EcoR1*, *AvaI*, *HindII,* and *TaqI* restriction enzymes were selected for the RFLP analysis. For this aim, 10 µl of PCR product, 2 µl of 10X enzyme buffer, and volumes of 1, 2, and 3 µl of enzymes (8 -10 units) were mixed together with DW to reach the final volume to the 20 µl. Next, incubation time periods of 4, 10, and 16 hours were considered at 37°C[15]. Finally, all PCR products, either mixed with restriction enzymes or not, were loaded onto the agarose gel wells and ran for 90 minutes at ambient temperature. At last, bands were visualized and captured using a Gel Doc apparatus[12].

3. Results

In the current study, a total number of 40 sheep were sampled from heart, scapular, esophageal and diaphragmatic muscles, among which 20 samples contained macroscopic cysts and 20 specimens were contaminated with microscopic cysts. In other words, 100% of examined muscles using the digestion method were infected with *Sarcocystis* spp. Following amplification of 18S rRNA of Sarcocystis DNA using specific primers, a 609 bp band was observed (Figure 3).

The *EcoRI* enzyme cut DNA-positive samples related to *S. tenella* and *S. arieticanis* but digests *S. gigantea* in position 644 bp. The *TaqI* enzyme cut *S. tenella* samples in positions 233 and 275 bp while digesting the *S. arieticanis* samples in positions 48, 58, 192, and 208 bp. These enzymes cut the specific sequences in *S. gigantea* amplified DNA in positions 270 and 338 bp. The *HindII* enzyme did not cut DNA samples related to *S. tenella* and *S. arieticanis*, while digested DNA of *S. gigantea* in 198 and 411 bp positions. The *AvaI* enzyme digested *S. gigantea* samples in positions 234 and 108, while it did not cut *S. tenella* and *S. arieticanis* DNA sequences (Table 1). All obtained bands following electrophoresis were visualized using a GelDoc apparatus.

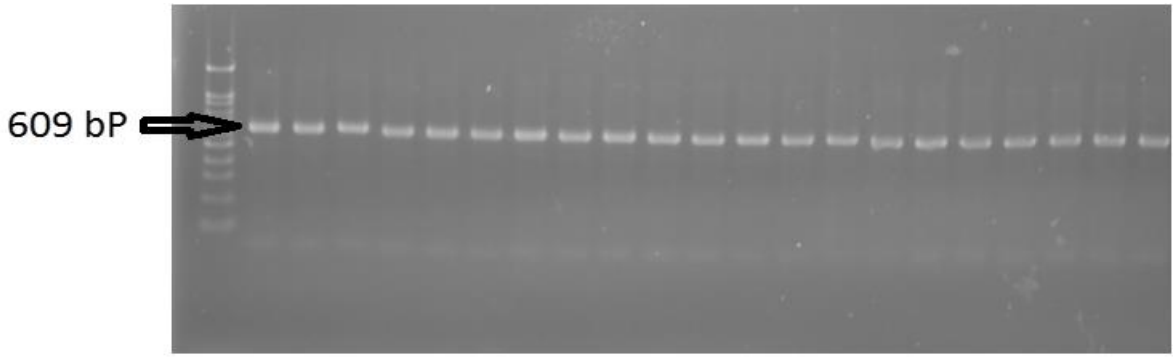

Figure 3. 18S rRNA PCR product analysis showing 609 bp bands representative of *Sarcocystis* genus

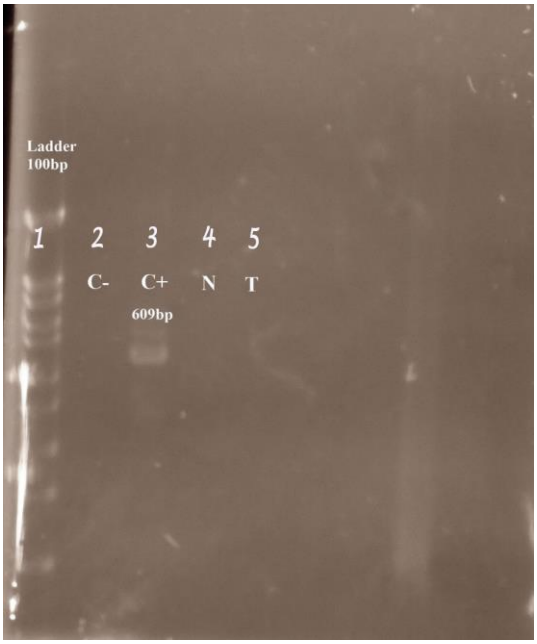

Figure 4. 18S rRNA PCR product analysis showing 609 bp bands positive for *Sarcocystis* genus. From left to right: 1. DNA marker; 2. Negative control; 3. Positive control; 4. *Neospora*; 5. *Toxoplasma*

Table 1. Frequency of *Sarcocystis* species in collected samples

	G	T	A	G+A+T	T+A	G+T	G+A
Total number of genotyped samples	20	30	42	0	15	20	20
percentage	33.3	50	70	0	25	33.3	33.3

G: *gigantean,* A: *arieticanis*, T: *tenella*

4. Discussion

Previously, sarcocystosis was considered a prevalent, innocuous disease of livestock, rendering inappropriate marketability of the infected carcasses[9]. Ovine sarcocystosis is reported in many countries across the globe with very high prevalence rates ranging from 9 to 100%[11]. Since the study demonstrated a high prevalence of sarcocystosis infection by microscopic and macroscopic cysts forming species, sheep could be considered alternative intermediate hosts for Sarcocystis[13]. The prevalence of macrosarcocysts has been reported in slaughtered sheep of different parts of Iran, including Fars Province in the south (57.70%), Shahrekord in the southwest (18.63%), Khoram Abad in

the west (6.67%), Kerman in the south (3.58%), in the north of Khorasan province in the northeast (0.04%), and Ahvaz in southwestern Iran (0.0049%). The reason for the high infection rate in intermediate hosts is the rearing of farm animals in close contact with carnivores which contaminate pastures with Sarcocystis sporocysts[14]. However, in recent years, sarcocystosis has been the focus of researchers as a well-recognized, frequent parasitic disease, as the causative agent of several health consequences in livestock (cattle, goat, sheep, and pig), such as fever, anemia, abortion, encephalomyelitis, lameness, paralysis, decreased milk production, muscle pains and finally mortality[2,8]. Nowadays, molecular techniques, such as PCR and RFLP are utilized for the definitive diagnosis of *Sarcocystis* spp. in animals and humans. 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, however, according to recently performed studies, cox1 is better for Sarcocystis species identification in sheep.

Among 4 species, *S. tenella* and *S. arieticanis* produce microscopic cysts and have pathogenic subsequences as acute disease presented by abortion, fever, anemia, and anorexia in the early period of infection, and then some chronic disorders maybe develop. On the other side, macrocyst forming species, S. *gigantea,* and *S. medusiformis* are considered nonpathogenic, but they can affect the meat quality and marketing and cause economic loss. Sheep may be infected with these four species simultaneously. This rate of microscopic infection in sheep in this area also indicates a high exposure rate of these animals to the sporocysts, which are shed by dogs and possibly wild animals that can be served as the definitive hosts. 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. In contrast, *S. gigantea* and *S. medusiformis* are nonpathogenic, while they may cause low-quality meat and economic losses[16]. 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, S. medusiformis, S. arieticanis,* 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%), and Iran (33.9%)[16]. Based on the results of the limited study, *S. tenella* and *S. gigantea* are present among the studied sheep in Isfahan province[16]. In Lorestan and Khuzestan provinces, Iran, it has been reported that all Sarcocystis isolated from slaughtered sheep belonged to *S. gigantea*[16].

The findings of the current study revealed a high prevalence of microsarcocysts infection in sheep. The low rate of macrosarcocysts in comparison with the microsarcocysts in the examined animals may be due to the rare contact between sheep and cats in the region. It was revealed that the abundance of macroscopic and microscopic Sarcocystis was 4.10% and 97.00% in investigated Iraqi sheep, respectively[6]. The high prevalence rate of microscopic Sarcocystis in sheep indicates the importance of the infection for the intermediate hosts[12]. The sex of examined sheep had a significant effect on the prevalence. However, in another study, it was reported that ewes had a higher prevalence of infection (7%) than males (93%)[8]. In addition, the prevalence of Sarcocystis infection in sheep increased as the age of the animals increased[12].

The results of the present study showed that sheep samples were contaminated by *S. tenella*, *S. gigantea,* and *S, ariteicanis*. In a study on sheep in northwest Iran, *Sarcocystis gigantea* was the only species isolated from sheep[14].

The results of the present study were similar to the previous study as the macroscopic cysts in Ziyaran slaughterhouse belonged to *S. gigantea* and the microscopic cysts belonged to *S. tenella* and *Ariti canis*[17].

Based on the current results, all specimens contained bradyzoites in the microscopic method, which is consistent with other investigations. For instance, 34.28% prevalence of *Sarcocystis* infection in slaughtered sheep in Mashhad abattoir using macroscopic observations, and an 81.9% positive samples in histopathology method; the prevalence and 100% in northeastern Iran has been reported[17]. The authors finally declared a considerably high prevalence of the infection among sheep in northeastern Iran. Moreover, *S. tenella* was the most frequent species in the area.

The first PCR-RFLP analysis in slaughtered sheep in Qazvin province, Iran, remarked that 100% of samples examined by digestion and stamp smear methods were positive, among which 40 specimens contained macroscopic cysts and 20 samples had microscopic cysts. Furthermore, 20 samples were related to *S. tenella* and *S. arieticanis*. Moreover, *S. gigantea* and *S. medusiformis* were detected in macroscopic analysis[18].

The genotype investigation of 10 tissue samples indicated that the macroscopic genotype was related to *S. gigantea* and the microscopic cyst was related to *S. tenella*[17]. The results of the current study revealed that microscopic cysts are more common than macroscopic cysts and can cause economic losses and cause many health consequences.

5. Conclusion

In the present study, 100% of samples were infected with bradyzoites using the microscopic method. Moreover, PCR-RFLP analysis indicated the presence of *S. gigantea*, *S. tenella,* and *S. arieticanis* among examined sheep. Pathogen species should be considered as the main agants of economic loss in livestock animals. Therefore, the implementation of further complementary studies is suggested in order to improve public health and enhance better recognition of sarcocystosis epidemiology.

Declarations

Competing interests

Authors declared no conflict of interest.

Authors' contribution

Ghazaaleh Adhami designed the study, wrote the draft of the manuscript, and did the statistical analysis. Samaneh Shakeri did sample collection, performing the molecular technique PCR-RFLP. The authors checked and approved the final version of the manuscript for publishing in the present journal.

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