Molecular determination of abundance of infection with *Sarcocystis* species in slaughtered sheep of Urmia, Iran

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**Abstract**

*Sarcocystis* is one of the most prevalent parasites of domestic ruminants worldwide. This study was aimed to determine prevalence of *Sarcocystis* infection and molecular discrimination of *Sarcocystis gigantea* and *Sarcocystis medusiformis* infecting domestic sheep. Tissue samples from 638 sheep slaughtered at Urmia abattoir were randomly collected from February 2011 to January 2012. Genomic DNA extraction and polymerase chain reaction (PCR) was performed to amplify a 964 bp fragment of nuclear 18S rRNA gene. The PCR products were subjected to digestion with endonuclease *MboI* and/or *MvaI* for discriminating *S. medusiformis* and *S. gigantea*. Results indicated that the overall prevalence of *Sarcocystis* unspecified species was 36.83% (235/638) in which male (7.63%, 38/498) and female (35.00%, 49/140) sheep over 4 years-old had the highest prevalence. There was no significant difference between prevalence of macrosarcocysts and sex. Two macrosarcocysts forms were found as fat (27.90%, 178/638) and thin (8.93%, 57/638) in striated muscles. There was significant difference between frequency of macrosarcocysts and body distribution. Mixed infection with both fat and thin macrosarcocysts was also found in 11.13% (71/638) of infected sheep. There was no significant difference regarding the prevalence of mixed infection in both age classes. The PCR-RFLP patterns showed that fat sarcocysts were *S. gigantea* (29.31%, 187/638) and thin sarcocysts were *S. medusiformis* (7.52%, 48/638). It was concluded that ovine *Sarcocystis* infection was prevalent in Urmia and a combination of conventional methods and molecular study for sheep sarcocysts could be informative.

**Keywords:** Molecular analysis, *Sarcocystis*, Sheep, Urmia
Introduction

The genus Sarcocystis (Lankester, 1882) is an obligatory intracellular and widely distributed protozoan of the phylum apicomplexa in a broad range of vertebrates’ livestock and humans with about 130 species. The parasite is heteroxenous and has an obligatory two hosts, i.e. intermediate host with merogony and cyst formation in skeletal muscles (sarcocysts) of herbivores or omnivores and definitive host with sporogony and gamogony in carnivores or omnivores.

Many animals are infected with one or more species of Sarcocystis. Sheep are intermediate hosts for S. gigantea (Railliet, 1886, syn. S. ovifelis), S. tenella (Railliet, 1886, syn. S. ovicanis), S. arieticanis (Heydorn, 1985), and S. medusiformis (Collins, Atkinson and Charleston, 1979). S. gigantea is distributed throughout the world while S. medusiformis has been found only in Australia, New Zealand, and Iran. Two of these species, S. gigantea and S. medusiformis are transmitted by felids and are non-pathogenic which develop macrocysts in striated muscles of sheep. Macroscopic cysts of these two Sarcocystis species are considered as causes of economic losses in the sheep industry. The heavily infected sheep meat by macrocysts in Iran may be condemned as unfit for human consumption. Furthermore, nearly all investigations on ovine Sarcocystis infection are limited to the slaughterhouse inspections without determination the prevalence of Sarcocystis species involved. The conventional method of distinguishing Sarcocystis species and combining these data with information on the life cycle are not suitable as a result of little morphological variation, high antigenic cross-reactivity, and time consuming.

Therefore, the small subunit (SSU) rRNA gene has been extensively used to differentiate between apicomplexans and other eukaryotic species due to its abundance in the genome and its double feature of hypervariable regions interspersed within highly conserved DNA sequences. Therefore, sensitive and specific molecular studies have been recommended to support detection and differentiation the parasites in the intermediate hosts that considered as a powerful tool for species-specific differentiation of ovine Sarcocystis species. In addition, there is no report concerning combine use of conventional methods and molecular techniques for comparison of Sarcocystis species in Iranian sheep. Therefore, the present study was carried out to determine prevalence of fat and thin macrocysts and molecular discrimination among Sarcocystis infecting sheep of northwestern Iran.

Materials and Methods

Animals. During the course of this study from February 2011 to January 2012, tissue samples (esophagus, diaphragm, and skeletal muscles) were randomly collected from 638 (498 male and 140 female) sheep slaughtered at the Urmia abattoir. All animals appeared healthy before being slaughtered. The age was estimated on the basis of eruption of permanent incisor teeth and the sex of each animal was recorded. The animals were categorized into two age classes, less than 4 years-old (n = 421) and over 4 years-old (n = 217), (Table 1).

Macromolecular and microscopic examinations. The esophagus, diaphragm and skeletal muscles were thoroughly inspected for the presence of Sarcocystis macrocysts. Specimens containing macrocysts were separated, the cysts were excised from the tissue, and classified in situ based on their characteristic, namely size, shape and location, (Fig. 1).

Pepic digestion method. Twenty gram of pooled muscles was incubated for 30 min at 40 °C in 50 mL of acid pepticin as digestion medium. The digestate was filtered through a fine meshed sieve into a tube, centrifuged at 2000 g for 5 min, and the sediment suspended in 0.5 mL of distilled water. The suspension was then microscopically examined for the presence of Sarcocystis bradyzoites under the light microscope at 400× magnification. In addition, further drops from the same solution were spread on glass slides, fixed, and stained with 1% Giemsa stain. The bradyzoites of Sarcocystis were measured using a micrometer eyepiece at 400× and 1000× magnifications.

DNA extraction. For molecular analysis, soft cysts of the macrocysts were dissected, washed several times in 0.01 M phosphate-buffered saline (pH 7.2), and stored at −20 °C until DNA extraction. Genomic DNA was isolated by modified phenol-chloroform method and stored at −20 °C.

PCR-reaction and RFLP analysis. A pair of primers, Sar-sense: 5'- TTCTATGGCTAATACATGGG-3' and Sar-antisense: 5'- CCAATACCTTCAAGAGA -3' were used to amplify a 964 bp fragment of the 18S rRNA gene of Sarcocystis. The PCR-reaction was carried out in 25 μL reaction mixture containing 5 μL (100 ng) of genomic DNA (diluted 1:30), 0.3 μL of Taq DNA polymerase (Fermentas, Heidelberg, Germany), 0.6 μL of 10mM dNTPs (CinnaGen, Tehran, Iran), 0.7 μL of 50 mM MgCl2, 2.5 μL of PCR reaction buffer (10×), 1 μL of each primer (25 mM), and 13.9 μL of distilled water. The reaction was performed in a Bioer XP thermal cycler (Bioer Technology Co., Tokyo, Japan). The samples were subjected to an initial denaturation step at 94 °C for 5 min, followed by 35 cycles of 60 sec at 94 °C, 40 sec at 57 °C, and 60 sec at 72 °C, and a final extension step at 72 °C for 5 min. A total volume of 10 μL of each PCR product was analyzed by electrophoresis on a long with positive and negative controls on TgDNA polymerase (Fermentas, Heidelberg, Germany), 0.6 μL of 10mM dNTPs (CinnaGen, Tehran, Iran), 0.7 μL of 50 mM MgCl2, 2.5 μL of PCR reaction buffer (10×), 1 μL of each primer (25 mM), and 13.9 μL of distilled water. The reaction was performed in a Bioer XP thermal cycler (Bioer Technology Co., Tokyo, Japan). The samples were subjected to an initial denaturation step at 94 °C for 5 min, followed by 35 cycles of 60 sec at 94 °C, 40 sec at 57 °C, and 60 sec at 72 °C, and a final extension step at 72 °C for 5 min. A total volume of 10 μL of each PCR product was analyzed by electrophoresis on a long with positive and negative controls on 1.5% (w/v) agarose gel for approximately 90 min at 80 V and visualized by staining with ethidium bromide.

To discriminate S. gigantea and S. medusiformis, RFLP method was employed. A total volume of 15 μL of digestion reaction containing 5 μL of the PCR product, 1 μL (1 U) of each of the restriction enzymes (Mbol and/or Mval,
Fermentas, St. Leon-Rot, Germany), 1 μL of enzyme buffer (Fermentas, Heidelberg, Germany), and 8 μL double distilled water was prepared. The reaction tubes were incubated at 37 °C for 16 hr. The digested PCR products were run on 2% (w/v) agarose gel and visualized by ethidium bromide staining. Digested products for S. gigantea by MboII was expected to generate 722 and 242 bps in length, while MvaI was expected to produce two fragments of 901 and 63 bps in size. Digested products by MboII and MvaI was respectively expected to generate 901 bps in size and undigested product for S. medusiformis.

Table 1. Prevalence of macro-sarcocysts and micro-sarcocyst of Sarcocystis species in slaughtered sheep at Urmia abattoir, Iran.

| No. of examined sheep | Muscle inspection (n/N, %) | PD (%) | MS (%) |
|-----------------------|-----------------------------|--------|--------|
|                       | Sex                        | Age (year) | Infected organs |                      |        |        |
|                       | Male | Female | < 4 | > 4 | Esophagus | Diaphragm* | Skeletal muscle |                |        |        |
| 638                   | 7.63 | 0 | 9.4 | 19.75 | 10.66 | 17.08 | 1.72 | 78.68 | 55.17 |
|                       | 0 | 35 | 0.16 | 7.52 | 1.72 | 4.55 | 1.41 | 17.71 | 15.2  |
| Total                 | 7.63 | 35 | 9.56 | 27.27 | 12.38 | 21.63 | 3.13 | 96.39 | 70.37 |

* indicates significant differences at p<0.05; PD: Peptic digestion method; MS: Muscle squash method; n: Animals infected with Sarcocystis; N: Total examined animals.

Results

Macroscopic and microscopic findings. The prevalence of macroscopic and microscopic sarcocysts in slaughtered sheep has been shown in Table 1. The overall prevalence of Sarcocystis unspecific species, including macroscopic sarcocysts in slaughtered sheep was 36.83% (235/638). In rams and ewes, the prevalence of macroscarcocysts were 7.63% (38/498) and 35.00% (49/140), respectively (p > 0.05). Among the different examined organs, macro-sarcocysts were found to be the highest in the diaphragm (17.08%, 109/638) and the lowest in the skeletal muscles (1.72%, 11/638), (p < 0.05).

The macro-sarcocysts occur as elongated cylindrical bodies and milky-white colored cysts embedded in the muscular tissues with length ranged from <5 mm to >10 mm (Fig. 1). Two inspected forms of macro-sarcocysts were fat (27.90%, 178/638) and thin (8.93%, 57/638) macro-sarcocysts in striated muscles of which were large enough to discriminate by naked eye. Fat macro-sarcocysts were in the diaphragm with at least mean length of 5-10 mm (range: 2.50 to 15.00 mm, n = 100). The cysts were fully packed with banana shaped bradyzoites averaging 5.43 × 22.36 μm (range: 3.16 to 7.38 × 18.77 to 27.69 μm, n = 100), (Fig. 1). Thin macro-sarcocysts occurred in esophagus and diaphragm with a mean length of 3.2 mm and width of 1.30 mm (range: 1.10 to 6.40 × 0.80 to 1.90 mm, n = 100). Also the bradyzoites of thin cysts were slightly smaller, averaging 4.21 × 17.29 μm (range: 2.95 to 6.38 × 16.41 to 23.15 μm, n = 100). Mixed infection with both fat and thin macro-sarcocysts was also found in 11.16% (71/638) of infected sheep in both age classes (p > 0.05). Regardless of the age and sex, the rate of micro-sarcocysts infection was found to be 70.37% (449/638) by muscle squash (MS) method. In male and female examined sheep, the prevalence was 55.17% (352/638) and 15.20% (97/638), respectively, (Table 1).

PCR-RFLP findings. Of both identified Sarcocystis species (Fig. 2), 29.31% (187/638) fat sarcocysts were S. gigantea (Fig. 3). It was also shown that the thin sarcocysts which were less frequent (7.52%, 48/638) than the fat sarcocysts were S. medusiformis, (Fig. 3). The highest prevalence was 19.75% (126/638) in ewes (> 4 years-old) infected with S. gigantea and it was also 3.45% (22/638) in male sheep (> 4 years-old) which were infected by S. medusiformis, (Table 2).

Table 2. Prevalence and diversity of non-pathogenic Sarcocystis species (%) in both sexes and age classes of slaughtered sheep by using PCR-RFLP, (n = 638).

| S. gigantea | S. medusiformis |
|-------------|----------------|
| Sex (%)     | Male | Female | Male | Female |Male | Female |
| Age (year)  | < 4 | > 4 | < 4 | > 4 | < 4 | > 4 | < 4 | > 4 |
| Prevalence  | 3.76 | 19.75 | 5.80 | 21.63 | 3.45 | 16.30 | 0.16 | 1.72 |
| Total       | 23.51 | 5.80 | 5.64 | 1.88 |

Summary

This study investigated the prevalence of macroscopic and microscopic sarcocysts in slaughtered sheep at Urmia abattoir, Iran. The overall prevalence of macroscopic sarcocysts in slaughtered sheep was 36.83% (235/638). The highest prevalence was found in diaphragm (17.08%, 109/638) and the lowest in skeletal muscles (1.72%, 11/638). The macro-sarcocysts occurred as elongated cylindrical bodies and milky-white colored cysts embedded in muscular tissues with length ranging from <5 mm to >10 mm. Two forms of macro-sarcocysts were fat (27.90%, 178/638) and thin (8.93%, 57/638). Mixed infection with both fat and thin macro-sarcocysts was also found in 11.16% (71/638) of infected sheep in both age classes. The prevalence was 55.17% (352/638) and 15.20% (97/638), respectively. The PCR-RFLP method was used to identify the species of sarcocysts, with 29.31% (187/638) being S. gigantea and 3.45% (22/638) being S. medusiformis.
The prevalence of macrosarcocysts has been reported in slaughtered sheep of different parts of Iran including Fars province in south (57.70%), Shahrekord in southwest (18.63%), Khoram Abad in west (6.67%), Kerman in south (3.58%), in north of Khorasan province in northeast (0.04%), and Ahvaz in southwestern Iran (0.0049%).

The prevalence of Sarcocystis infection in sheep increased as the age of animals increased. The age related distribution of Sarcocystis infection in age group <4 years-old was similar to that reported previously by Oryan et al. Also, molecular differentiation of ovine sarcocysts species showed the same pattern of age related prevalence of Sarcocystis infection for both identified Sarcocystis species. This difference might be due to the much higher mean age of male sheep at the time of slaughter and possibility of sarcocysts growing and high sensitivity of older sheep to gain infection.

The morphology of fully developed macrosarcocysts in the intermediate host varies among different Sarcocystis species and has been previously used to differentiate the species. In current study, two forms of macrosarcocysts were found in sheep muscles which were respectively discriminated as S. gigantea and S. medusiformis using molecular examination. Fat macrosarcocysts were commonly found in the diaphragm, while thin macrosarcocysts were exclusively found in both esophagus and diaphragm of examined sheep. According to Oryan et al. and Heckeroth and Tenter, S. gigantea is predominantly found in oesophagus, larynx, and lingua muscles. They were also reported that S. medusiformis is commonly found in diaphragm, abdomen, and skeletal muscles.

The results of a number of epidemiological studies on Sarcocystis infections in Iranian sheep by peptic digestion method (PD) procedure were in agreement with the results in the present study. The PD procedure gave the highest rate of infection and it was found more sensitive, simple, and rapid than tissue sectioning for detecting sarcocyst infections.

In current study, molecular investigation also evidenced both identified fat and thin sarcocysts respectively belonged to S. gigantea and S. medusiformis. It was also shown that the S. medusiformis were less frequent than S. gigantea in infected carcasses. Molecular findings indicated that the highest prevalence was in ewes infected with S. gigantea and male sheep infected with S. medusiformis over 4 years-old. In recent years, the advent of new molecular biological techniques has provided new diagnostic means for parasitic infections. The ssu rRNA
gene amplification is specific way for discriminating Sarcocystis species.\(^{35}\) In earlier studies, PCR-RFLP method introduced as a tool with high specificity and susceptibility of discriminating Sarcocystis species worldwide.\(^{12,36-38}\) PCR-RFLP was applied for the first to identify S. gigantea in Iranian sheep and recommended as easy and rapid method than DNA sequencing of discriminating between these species by Dalimi et al.\(^{39}\)

In conclusion, the present work has demonstrated that Sarcocystis infection was common in sheep in Urmia. In addition, sheep husbandry is a sector of food supply for the rural and sometimes urban people and their health status is therefore important. Therefore, further investigations may reveal more information about economic effects of each type of this parasite in the region. On farms, it is important to reduce the sporocysts shedding in dogs and/or cats, which in turn will aid reduction of transmission within flocks. Finally a combination of conventional diagnostic methods and molecular identification of sarcocysts in sheep will be informative.

Acknowledgements

This study was supported financially by the Urmia Faculty of Veterinary Medicine, Urmia University, Urmia, Iran. The authors would like to acknowledge the support and interest of the technical members of the Urmia abattoir and Department of Pathobiology, especially, Armen Badali, Urmia University, Urmia, Iran.

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