Prevalence and Concentration of *Sarcocystis* spp. Microscopic Cysts in Sheep Muscles Using Percoll Gradient Centrifugation

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ABSTRACT

Percoll gradient centrifugation can purify different parasitic stages from tissues or faeces of hosts. *Sarcocystis tenella* and *Sarcocystis arieticanis* develop microscopically visible cysts in sheep muscles. In this study, the distribution of microscopic cysts were determined in different muscles groups of sheep naturally infected with *Sarcocystis* spp. by using percoll gradient centrifugation. *Sarcocystis* spp. microscopic cysts were detected as 91% of sheep. *S.tenella* and mixed infections with *S.tenella* and *S.arieticanis* were observed in the tissue samples at a prevalence of 91% and 18.7%, respectively. *Sarcocystis* species cysts were most prevalent in the tongue muscle tissue at a rate of 80%. The cysts were observed a rate of 73%, 69% and 61% in the masseter, intercostal muscles and diaphragm, respectively. The relationship between the cysts present and the different muscles groups was significantly different ($p<0.001$). The number of microscopic cysts ranged from 4-476 (mean 235) per 5 grams muscle samples. Percoll gradient centrifugation should be considered as an alternative method for detection of *Sarcocystis* spp. microscopic cysts in host muscles.

Keywords: *Sarcocystis tenella; Sarcocystis arieticanis*; sheep; microscopic cyst; percoll gradient centrifugation.

INTRODUCTION

Sarcocystis species are obligatorily intracellular protozoa. Carnivorous animals are final hosts and herbivorous animals are generally intermediate hosts of these species. *Sarcocystis* spp. possesses a typical coccidian life cycle. Multiplication by merogony and cyst formation take place in intermediate host, gamogony and sporogony in the final host (1, 2).

Sheep are intermediate hosts of four *Sarcocystis* species. *Sarcocystis gigantea (sin. S.ovifelis) and Sarcocystis medusiformis* develop macroscopic cysts, *Sarcocystis tenella (sin. S. ovicanis)* and *Sarcocystis arieticanis* develop microscopically visible cysts in sheep muscles (1). The cyst wall structure is important for the identification of *Sarcocystis* species (2). *S.tenella* has villiar protrusions of about to 3.5 μm long and *S.arieticanis* have hair like projections 5-9 μm long in the wall (1, 2).

Infection with *Sarcocystis* spp. is prevalent in sheep worldwide (1). It is difficult to determine the pathogenicity of *Sarcocystis* spp. in naturally infected sheep as animals may be infected concomitantly by several *Sarcocystis* species of differing virulence (2). *S.tenella* can cause anorexia, weight loss, fever, anaemia, loss of wool, premature birth, nervous signs, myositis and death, depending on the number of sporocysts ingested (1, 3). Also, ovine abortion associated with *Sarcocystis* spp. has been reported (4). *S.arieticanis* is less pathogenic than *S.tenella* (1).

Several epidemiological studies on sarcocystosis in sheep have been carried out using different diagnostic methods (5-9). Percoll is a sterile colloidal suspension collected with silica particles of about 15-30 nanometres in diameter. During the centrifugation the Percoll suspension allows structures such

as cells, organelles and bacteria to be separated according to their density (10). Percoll gradient centrifugation is able to purify different parasitic stage from tissues or faeces of host such as *Toxoplasma gondii* tissue cysts from brain and *Cryptosporidium* spp. oocysts from faeces (11-13). To best of the authors' knowledge, no data has been reported about detection of *Sarcocystis* spp. microscopic cysts using percoll gradient centrifugation.

The aim of the study was to determine concentration and distribution of microscopic cysts in different muscles groups of sheep naturally infected with *Sarcocystis* spp. by using percoll gradient centrifugation.

MATERIAL AND METHODS

Collection of tissue samples

Sampling was carried out in slaughterhouses of Kirikkale province, located in the Central Anatolia region of Turkey during 2011. After slaughter, skeletal muscles (tongue, diaphragm, masseter, limb and intercostal) of Akkaraman sheep (n=100) (77 male and 23 female) were sampled into separate sterile bags.

Percoll gradient centrifugation

Five grams of tissue samples from each muscle were cut with sterile scissors and added to 20 ml of PBS. Then, the samples were homogenised using a high speed tissue homogeniser (OMNI Tip, USA). The homogeniser was washed in boiled water between every consecutive homogenization. The homogenates were filtered into a centrifuge tube using separate cheesecloth. Percoll stock solution was diluted to 90% and 30% with distilled water and NaCl (for 90% Percoll dilution; 8 ml of Percoll stock solution + 1 ml of distilled water + 1 ml of 1.5M NaCl, for 30% of Percoll dilution; 3 ml of Percoll stock solution + 6 ml of distilled water + 1 ml of 1.5M NaCl). The Percoll dilutions and the homogenates were centrifuged at 4,000 x *g* for 20 min. Following centrifugation, the different Percoll layers were placed on slides using a Pasteur pipette and examined for presence of tissue cysts using a binocular light microscope (Olympus BX 50, Japan).

Statistical analysis

Mann Whitney U test was used to analyse the differences between the sexes in regard to the presence of microscopic cysts. The Kruskal Wallis test was used to compare the differences among sampled muscles and ages of sheep for the presence of microscopic cysts. Statistical analysis were carried out using the statistical software package SPSS, version 15.0. *P* values less than 0.05 were considered significant.

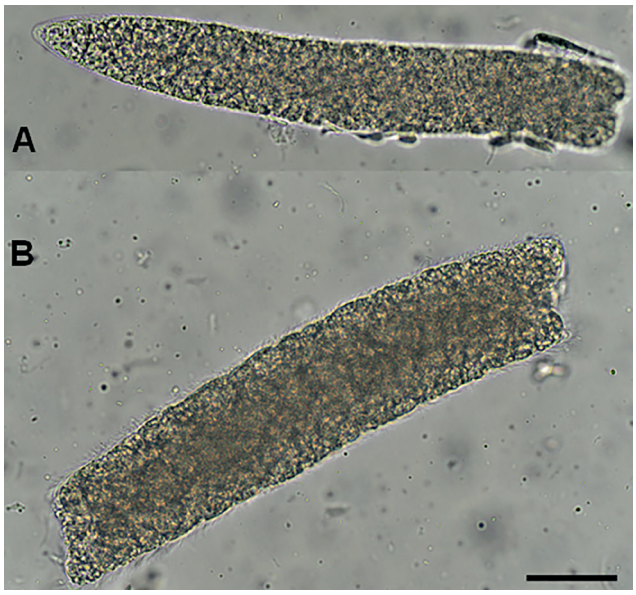

Figure 1: Microscopic cysts of Sarcocystis spp. in sheep: A. *S.tenella*, B.*S.arieticanis* with hair like projections in the wall (Bar: 50 µm).

RESULTS

Sarcocystis spp. microscopic cysts were detected in 91% of the sheep examined. *S.tenella* and mixed infections with *S.tenella* and *S.arieticanis* were detected in the muscle samples at a rate of 91% and 18.7%, respectively (Figure 1). The tongue muscles showed the highest detection rate for *Sarcocystis* spp. cysts (80%). The cysts were observed as 73%, 69% and 61% in the masseter, intercostal muscles and diaphragm, respectively. The relationship between the cysts present and the muscles groups was statistically significant (p<0.001).

Table 1: The relationship for the presence of Sacocystis microscopic cysts in the muscles of sheep by age and sex.

	Sheep	Sarcocystis spp. positive		
Sex	Age	Number (n:100)	Number (n:91)	%
Male	1	50	41	82
	2	19	19	100
	3	5	5	100
	4	3	3	100
Female	1	13	12	92
	2	2	2	100
	3	7	7	100
	4	1	1	100

The cysts were observed in 88.3% (68/77) of rams and 100% (23/23) of ewes ($p>0.05$). Furthermore, *Sarcocystis spp.* cysts were detected in 85.7% (54/63) of sheep aged 1 year and 100% of sheep aged from 2 to 4 (37/37) ($p<0.01$) (Table 1). The number of cysts ranged from 4-476 (mean 235) per 5 gram muscle samples.

DISCUSSION

Sheep sarcocystosis is prevalent worldwide (5-9). Sarcocystis species transmissible by cats has been found less frequently than those transmissible by canids (1). Feline-transmitted species require long periods of time to become infective in intermediate hosts (2).

Sheep become infected with *S. tenella* or *S. arieticanis* by ingesting sporocysts in contaminated food or water. The asexual development of both species consists of two generations of endopolygeny in vascular endothelial cells. Endozoites initiate the formation of cysts in various groups of striated muscles (1, 2). Maximum cysts numbers have been reported in tongue muscle of intermediate hosts (8, 14). In the present study, the tongue was the most prevalent tissue for *Sarcocystis* spp. microscopic cysts. The relationship between the presence of cysts in the different muscles groups was statistically significant ($p<0.001$). The preferential distribution of *Sarcocystis* spp. cysts in the tongue may possibly be related to the greater circulation of blood to these muscles.

It has been claimed that the prevalence of *Sarcocystis* spp. microscopic cysts is not related to age (15). In our study, *Sarcocystis* spp. cysts were more prevalent in sheep aged 2-4 year compared sheep of 1 year of age (100% vs. 87.5%). The relationship between the prevalence and the age was found to be statistically significant ($p<0.01$). The result presented herein suggests that exposure to sporocysts of *Sarcocystis* spp. can cause an increase in the muscles of sheep with age.

Based on clinical signs, diagnosis of sarcocystosis is very difficult (1). Macroscopic cysts can be seen with the naked eye in carcasses of sheep after slaughter. However, microscopic cysts can only be detected by more refined diagnostic techniques. The cyst wall structure is used to identify the *Sarcocystis* spp. (2). Studies have been reported based on microscopic examination after the enzymatic digestion of muscles samples of intermediate hosts (16-18). This method may result in difficulties in identification of *Sarcocytis* species as the cyst's wall may be partially digested by the enzymes resulting in the release of cystozoites which cannot be distinguish from each other due to their similar morphology (1, 19).

Another diagnostic method is histopathology. Cysts can be seen in stained tissue sections. However, *Sarcocystis* spp. microscopic cysts can be confused with tissue cysts of other protozoan parasites such as *T.gondii*. Immunohistochemical staining is sometimes necessary to distinguish between parasite species (1). Electron microscopy can be used to differentiate of *Sarcocystis* spp. according to their wall structure (20). However, specialized technical personnel and equipment are required for this analysis. Compression is another technique used to detect *Sarcocystis* spp. cysts in intermediate host tissues (8). In this method muscle pieces are squashed between two glass slides and examined for cysts by light microscopy (8). However, small numbers of cysts cannot be detect because a small amount of tissue is examined using this method.

In addition to the methods mentioned above, PCR is used to detect parasite DNA in tissue samples (21, 22). Molecular technique is not always suitable in epidemiological studies due to the high expense. In addition the PCR method is disadvantageous due to the small amount of tissue examined by this method because *Sarcocystis* spp. microscopic cysts are distributed randomly in host tissues.

In the present study, the Percoll gradient centrifugation was used to determine *Sarcocystis* spp. microscopic cysts in sheep muscles. The method was suitable for the release of *Sarcocystis* spp. cysts from muscles cells and allows for morphologic identification of the species responsible for infection.

In conclusion, *Sarcocystis spp.* microscopic cysts were detected in nearly all of sheep examined in the present study. The cysts were mostly detected in tongue muscles and the relationship between the cysts present and the muscles groups was significant statistically ($p<0.001$). The Percoll gradient centrifugation should be considered as an alternative detection method of *Sarcocystis* spp. microscopic cysts in muscles of intermediate hosts. Also the method allows quantification of cysts per gram tissue sample.

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