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Evaluation of Sarcocyst Parasite Strains in Carcasses Obtained from Ardabil Meat Industrial Group

Ciamak Ghazaei

Department of Microbiology, University of Mohaghegh Ardabili, Ardabil, Iran, P.O. Box 179

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ABSTRACT

Sarcocystosis is a common protozoan infection in humans and animals which is caused by various species of Sarcocystis. Hence, the aim of this study was to evaluate strains of Sarcocystis parasites in carcass of 250 cattle and 250 sheep slaughtered in the Ardabil Meat Industrial Group. Carcasses were compared by macroscopic and digestion techniques, staining and non-staining tissue smear and molecular PCR-RFLP. The organs examined were diaphragm, intervertebral muscles, esophagus, heart and tongue. The results were analyzed using Chi-square test. Out of 250 cattle and sheep examined, 28 and 124 cases had macroscopic cysts and 162 and 211 cases had microscopic cysts, respectively. Of 28 cases of macroscopic cysts in cattle, 21 cases of S. hominis and 7 cases of S. hirsuta were diagnosed. In 100% (162) of bovine specimens containing microscopic cysts, S. cruzi was diagnosed. In 100% of sheep samples containing macroscopic cysts, S. gigantea was diagnosed. In 211 sheep samples containing microscopic cysts, 156 cases of S. arieticanis and 55 cases of S. tenella were diagnosed. We concluded that the studied meat of the Ardebil Meat Industrial Group is highly infected with Sarcocystis parasite. Due to the health importance and infection rate of this parasite, it is recommended to avoid consumption of half-cooked and grilled meat from cattle and sheep.

1. Introduction

Sarcocystis is a genus of parasitic protozoa within the Sarcocystidae family. Infection by *Sarcocystis* leads to a disease called Sarcocystitis primarily due to a poison sarcocysteine secreted by this protozoan refers to a poison from sarcocysts. Meisher tubes name came from Meisher (1843), who reported first time Sarkosporiodia, in the form of white veins with a length of 1-3 cm in mice muscles. *Sarcocystis* is associated with intracellular protozoan of Apicomplexa (Dubey *et al.*, 1989). This parasite has a global spread (Fayer, 2004) and is one in every of the foremost common parasites in domesticated livestock, that causes very severe contamination in some hosts such as cattle and sheep. It also infects many wild animals, birds and cold-blooded animals (Mala and Baranova, 1995; Nevole and Lukesova, 1981) additionally, certain places of world also get infected by some species of this parasite including humans which leads to vital health implications (Fayer *et al.*, 2015; Tappe *et al.*, 2015). The final host, human is infected by some species of protozoa by uptake meat containing cysts in the muscle fibers, that is sarcocysts. These sarcocysts contains high amount of

*Corresponding author: Dr. Ghazaei
Tel: +98 045-33512081-9
E-mail address: ciamakghazaei@yahoo.com

bradyzoites. Bradyzoites is responsible for sexual development within the gut wall and becomes ovocyst; the sporozoitis containing sporozoite is excreted in faces from the final host and is injected by the intermediate host, i.e., cattle, sheep, goats and pigs and therefore the parasitic life cycle is completed (Dubey *et al.*, 2000).

Some species of *Sarcocystis* can cause disease and thus weight loss, loss of appetite, fever, anaemia, muscle weakness, reduced lactation, abortion, and sometimes cause animal death in intermediate hosts such as cattle and sheep (Herenda, 2000). *Sarcocystis* is a common parasite in many livestock. This intracellular protozoan parasite is an Apicomplexa (Valinezhad *et al.*, 2008) and can cause economic losses and clinical and subclinical illnesses. *Sarcocystis* has 130 heterogeneous species, with different cycles and pathogens. *Sarcocystis* pathogenic species is *Sarcocystis hominis* and *Sarcocystis suihominis*, in which humans act as certain hosts and cattle and pigs act as intermediate hosts. Infection with this parasite leads to health and economic harm to humans as well as animals (Mirzaei and Rezaei, 2016). *Sarcocystis* has two life cycles based on hunted-hunter relationship in a grassland or omnivorous as the intermediate host (hunted) and a carnivorous ultimate host (hunter) (Dalimi *et al.*, 2008). Studying *Sarcocystis* species, various species of this parasite have been reported in sheep around the globe. Some of these species, such as *S. gigantea* (ovifelis) and *S. medusiformis*, are macroscopic and others, such as S. *tenella* (ovicanis) and S. *arieticanis*, are microscopic (smaller than one millimeters) on carcass of sheep (Arshad et al., 2007). Three species of *Sarcocystis* have been identified in cattle. S. *hirsuta* in the muscles produce macroscopic cysts with hairy deformities; its final host is cat and it is mild pathogenic. *S. cruzi* produce microscopic cysts without hairy deformities; its final host is Canidae. This parasite is the most pathogenic species of *Sarcocystis* in the cattle (Arshad et al., 2007). To diagnose *Sarcocystis*, it is clinically implicated in both intestinal and muscular forms. *Sarcocystis* is a gastrointestinal tract parasite in the final host and muscle parasite in the intermediate host. Diagnosis depends on exact evaluation of the herd and its relationship with the dog and other final hosts associated with clinical symptoms. Diagnosis of humoral antibodies and histopathologic examinations include a wide range of organs, including kidneys, liver, spleen, muscle, lymph nodes, and spinal cord. Observation is usually used for diagnosis in the slaughterhouse; however, two laboratory methods can also be used: 1) digestion method; 2) smear (Fayer, 2004).

In terms of testing the transmission of this parasite, intermediate hosts are infected by eating contaminated feed or eating forage from the rangeland infected with sporozists exerted from the final hosts; the final hosts are infected by eating tissues infected by cyst of intermediate hosts. The size of cysts depends on the type of parasite species and host type. Cat is the final host for macroscopic form cysts and dog is the final host for microscopic form cysts (Dalimi *et al.*, 2008). The disease from various species of this protozoan is important for human health and economically; millions of dollars are annually lost by the livestock industry as a result of destruction of carcasses infected with sarcocyst (Hamidinejat *et al.*, 2010). Due to high percentage of livestock infection rate with this parasite and its medical and veterinary significance, this study tends to examine sarcocyst parasite strains by molecular method in cattle and sheep carcasses, and determined the prevalence of *Sarcocystis* infection in cattle and sheep carcasses obtained from the Ardabil Meat Industrial Group, and compare four different methods of carcass inspection by macroscopy, digestion method, staining tissue smear, non-staining tissue smear, and molecular PCR-RFLP in diagnosing meat infected with this parasite.

2. Materials and Methods

For this study, the Ardabil Meat Industrial Group was visited in different months of the year; 250 cattle and 250 sheep were randomly selected. Livestock characteristics such as age, gender, and race were recorded in the relevant questionnaire. The study included two steps. In the first step, slaughterhouse was visited, the carcasses were examined by direct observation of diaphragm, intercostal muscles, esophagus, heart and tongue for findings macroscopic cysts. In the second step, samples were taken from each of the above organs (samples were sterilized in a plastic sterile container); microscopic examination of the samples was

done by digestion method, staining tissue smear, non-staining tissue smear, and molecular PCR-RFLP. The following methods were used for experiments.

2.1. Digestion method

In this method, a standard protocol was used to make an artificial digestive solution used for studying tissue parasites, such as trichinosis and tissue protozoans (Rahimi *et al.,* 2002). After taking the muscle sample from the organs by sterile scalpel, scissors and forceps, connective tissues and fat were removed from the muscles; then, 20 g tissue was scaled and crushed with a meat grinder and dissolved in 50 ml digestive solution. The digestive solution contained 1.3 g pepsin, 2.5 g Sodium Chloride (NaCl), 3.5 ml hydrochloric acid in 500 ml distilled water. Then, the container containing the sample was placed in an incubator at 40°C for 1 h. After incubation solution was passed through a filter paper. Then, filtered solution was centrifuged at 2500 rpm for 5 min; it's deposition was examined by an optical microscope with a magnification of 400x for the presence of *Sarcocystis cystizoite* (Rahimi *et al.,* 2002).

2.2. Tissue smear

About 1 g of meat sample was placed between two glass slides and a thin smear was prepared by pressing it. Once the slide was dried at the laboratory temperature, smear was stabilized by methanol. First, non-staining slides were examined under light microscope. The stabilized slides were placed in a Giemsa staining solution for 17-22 minutes and examined under a microscope for presence of bradyzoite *Sarcocystis* (in this case, when cysts were ruptured by digestion, bradyzoites were seen in the form of parallel objects) with a magnification of 40x and 100x. By observing bradyzoite forms, the sample was considered as positive and recorded in the relevant table.

2.3. Molecular examination

After microscopic and macroscopic examination, intercostal muscle, tongue, diaphragm, heart, and esophagus infected with *Sarcocystis* were collected from sheep carcasses to perform molecular analysis of *Sarcocystis* parasite strains in sheep. *Sarcocysts* were carefully removed from the muscle by a scalpel and placed in separate containers. Then, the sarcocysts were first washed once by sterile normal saline and twice by buffer (10 mM Tris-HCL, 1 m EDTA) TE (pH 8). Subsequently, a DNA extraction kit (Sinagen) was used to extract DNA from tissues. For this purpose, the extracted DNA was diluted by distilled water (1:50) and its adsorption was determined at 280 and 260 nm wavelengths. Then, PCR was

performed to confirm *Sarcosystitis* (Bahari *et al*., 2014). In this method of regional proliferation, *Sarcocystis* genome encoding 18s rRNA unit was used using a specific primer (Table 1).

Table1. Forward and reverse primer sequences used in PCR

Name	Sequence from 5′ to 3′	Primer Length
Sar-F1	GCACTTGATGAATTCTGGCA	20 bp
Sar-R1	CACCACCCATAGAATCAAG	19 bp

Polymerase chain reaction (PCR) in 30 µL with thermocycler: initiation step pre-denatuation started with separating two primary target stands for 3 min at 94°C; 35 cycles involving: denaturation for 35 s at 95°C, annealing done with specific primer to the target for 45 s at 56°C, extension was performed for 1 min at 72°C, and at last final extension for 5 min at 72°C. Electrophoresis was performed by the end point of PCR to observe the bands. For sequencing PCR product was sent to the Iran Veterinary Organization. SPSS 16 software was used for data analysis.

3. Results

In this study, 250 cattle and 250 sheep in the Ardebil Meat Industrial Group were examined for Sarcocyst parasite. All carcasses were observed for macroscopic and microscopic cysts. Of 250 cattle examined, 9 cases had macroscopic cysts and 100% of samples had microscopic cysts. Of 250 sheep examined, 89 cases had macroscopic cysts and 211 cases had microscopic cysts. Meanwhile of 250 cattle studied, 87 were male and 163 were female; of 250 sheep studied, 94 were male and 156 were female; in both the animals infection rate with Sarcocyst parasite was higher in female cattle

and sheep compared with male cattle and sheep in both of digestion method and macroscopy (Table 2).

Of 9 cases of macroscopic cyst in cattle, 6 cases (66.66%) were *S. hominis* and 3 cases (33.33%) were *S. hirsuta*. In 100% (197) of samples, bovine specimens containing microscopic cysts were diagnosed with *S. cruzi*. In 100% (89) of samples, sheep specimens containing macroscopic cysts were diagnosed with *S. gigantean*. In 211 sheep specimens containing microscopic cysts, 156 (73.93%) were diagnosed with *S. arieticanis* and 55 (26.06%) were diagnosed with *S. tenella*. Infection rates of different organs of cattle and sheep are shown in Tables 3 and 4.

According to Table 3, macroscopy revealed that the most infected organ in 250 bovine specimens was diaphragm with 3 cases (33.33%). There was no significant difference in organs of the studied cattle in terms of infection with *Sarcocystis c*yst ($P<0.05$). In digestion method, infection with *Sarcocyst* parasite was diagnosed in 197 specimens (78.80%). According to Table 4, macroscopy revealed that the most infected organs examined in sheep was diaphragm with 32 out of 72 specimens (44.44%); there was a significant difference in sheep organs in terms of infection with *Sarcocystis* cyst ($P<0.01$). Also in digestion method, infection with *Sarcocyst* parasite was diagnosed in samples varying from 37.14% to 100%.

Table 2. Infection rate of *Sarcocystis spp.* by macroscopic and digestion methods in cattle and sheep by sex

Positive					**Number of tested cases**		
Female		**Male**					
%	N	%	N		Female	Male	
							Cattle
66.66	6	33.33	3	Macroscopy	163	87	
59.89	118	40.10	79	Digestion	163	87	
							Sheep
73.03	65	26.96	24	Macroscopy	156	94	
60.18	127	39.81	84	Digestion	156	94	

Table 3. Infection rate of *Sarcocystis* spp. in cattle's organs by used methods

Cattle						
Digestion			Macroscopic			
%	N	N	%	N	N	
22.84	45	55	11.11	1	55	Esophagus
25.88	51	65	22.22	2	65	Diaphragm
17.25	34	35	22.22	2	35	Heart
43.29	42	45	33.33	3	45	Tongue
12.69	25	50	2	1	50	Intercostal

* N: Number

Table 4. Infection rate of *Sarcocystis* spp. in sheep's organs by used method

Sheep						
Digestion			Macroscopic			
%	N	N	%	N	N	
82.35	56	68	35.27	19	68	Esophagus
100	72	72	44.44	32	72	Diaphragm
85.71	30	35	27.45	14	35	Heart
37.14	13	35	11.47	7	35	Tongue
100	40	40	34.69	17	40	Intercostal

*N: Number

Table 5. Infection rate of *Sarcocystis* spp. by sheep age

Macroscopic Infection			Infection age
Total	Negative	Positive	
Number/Percentage	Number/Percentage	Number/Percentage	
97	73	24	Less than 1 year
100	75/25	24/74	
153	88	65	More than 1 year
100	57/52	42/48	

According to Table 5, parasite infection was higher in sheep more than one-year-old.

4. Discussion

Due to health significance of this parasite, researchers have done various studies in different parts of Iran. In Iran, the first studies on diagnosis of *Sarcocystis* were done by Afshar et al. in 1974. This study was conducted by Shekarforosh and Ahmadi (2004) in Isfahan using contact expansion, 94.8% of the cattle were infected (Shekarforosh and Ahmadi *et al*, 2004). Razmi and Rahbari (2000) conducted a study on *Sarcocystis* infection in domestic ruminant populations of Tehran and Golestan provinces. In this study, infection rate of microcystic parasites were zero in cattle and infection with microcyst parasite was 79.73% (Razmi and Rahbari *et al*., 2000).

One of the reasons for the lack of macroscopic cysts in cattle is that the species which infect the cattle are their final host, often dog or Canidae; often these species cause microcysts in the intermediate host. High infection of cattle can be attributed to abundance of stray dogs and their movement on the farms as well as manual feeding of livestock and forage stored in the warehouse, which can keep the parasite away from the severe environmental conditions. Mohanty et al, 1995 reported an infection rate in female cattle and sheep increase, and the main reason was longer breeding and keeping time in female animals.

The results of many studies indicated that microscopic method is more preferable compared to macroscopic method. The reasons for low incidence of macroscopic cysts in other similar studies include:

1. Low frequency of S. felis compared to S. canis.
2. Fewer dispersion of cat stool than dog stool in rangelands (shepherd dog usually accompanies the animal herds).
3. Fewer distribution and number of cats than dogs.
4. Cats exert less sporocysts than dogs; cat sporocyst is more likely to need to survive in the environment for infection (Dehaghi *et al.*, 2013).

The most important factor in spread of disease is removal of sporocytes from the feces of the final hosts; thus, it is necessary to prevent the movement of these animals (dogs and cats) to cattle and sheep breeding canters. To prevent completion of life cycle of the parasite, carcasses of hosts (cattle and sheep) should be kept away from the final hosts (dogs and cats), so that dogs and cats do not have access to sarcocyst-infected meat; the infected meat should be either placed in special wells or disposed so that intermediate hosts cannot access it (Najafan *et al.*, 2008). Since slaughter house inspections only diagnose macroscopic cysts, rather than microscopic cysts (Najafan *et al.*, 2008), this method cannot be a suitable method for diagnosing infection. In order to

prevent infection with this protozoan, keeping meat at 100°C for 4 min results in destruction of microscopic *Sarcocystis* cysts or cooking at a temperature of at least 60°C for 20 min and 70°C for 15 min is recommended to prevent the disease. Moreover, freezing at 4°C for 48 h and -20°C for 24 h also results in destruction of bradyzoites (Alibeigi *et al.,* 2015). Several studies have shown that tissue digestion is more sensitive than other methods in diagnosis of *Sarcocystis* (Collins *et al.,* 1979).

In the current study, infection with *Sarcocystis* parasite was higher in female sheep compared with male sheep. Sheep older than one year has more chances to get infection compared with one year younger sheep. Arshad *et al.* (2007), showed that infection rate of female sheep was higher than male sheep; however, this difference is more related to sheep age because, unlike male sheep, older female sheep are slaughtered, which is consistent with the current study. Infection is more likely to occur in older ages due to longer exposure to develop the infection and form the cyst. Different species of sheep are completely different in terms of the shape and size of cysts. Due to small size and visibility of some cysts and severe infection of sheep, the meat should be cooked carefully regardless of normal inspection results (Dalimi *et al.,* 2008).

Currently, molecular methods are more preferable to diagnose sarcocystic species. This method can even be used to diagnose infection in alive livestock (Heckeroth and Tenter, 1999). *S. gigantea* or *S. ovifelis*, in addition to Iran, has a global spread. Its definitive host is domestic cat. S. *gigantic* cysts are found in esophagus, larynx, and tongue muscles and to a lesser extent in diaphragm and other sheep muscles. Sarcocysts does not exist in heart muscles and central nervous system. Macroscopic cysts are seen usually in older sheep compared with younger sheep. These cysts are up to 1 cm long, white opaque and elliptical spheroid or pear-shaped; sometimes, they resemble to rice seeds. *S. gigante* is a mild pathogen for sheep (Dubey *et al.,* 2004). However, it is very important in terms of meat inspection and may result in carcass capture in severe infection.

In most slaughter reports, livestock *Sarcocystis* infection is probably related to this parasite, because only this species and *S. medusiformis* produce large cysts in sheep and their cysts are visible. *S. tenella* and *S. rieticanis*, as two pathogenic and dangerous species, produce small and microscopic cysts in sheep muscles. These cysts are not visible to the naked eye. Due to speed and accuracy of PCR-RFLP results, primers considerably contribute in identifying various species of animal *Sarcocystis* in Iran (Dalimi *et al.,* 2008).

Finally, this study determined the infection rate and strains of sarcocyst strains in cattle and sheep carcasses in the Ardabil Meat Industrial Group. According to results our study, it was determined that infection rate of slaughtering cattle with macroscopic cysts of sarcocyst protozon was low. Although a lower percentage of bovine carcasses was infected with macrocysts of this protozoan, 100% of these carcasses contained microscopic cysts of this parasite. Infection of different tissues with this protozoan varies depending on anatomical position and blood supply to tissues. On the other hand, our study also showed high infection rate of slaughter sheep with sarcocyst; in this regard, infection rate to both macroscopic cysts and microscopic cysts is high. According to different studies conducted in the world and Iran, which used different methods to determine prevalence of *Sarcocystis* and reported 100% infection rate, and based on the results obtained for studying infection rate with sarcocyst parasite in slaughtered meat of the Ardabil region and with regard to prevalence of *Sarcocystis* infection and particularly microscopicity of the majority of cysts, the meat should be cooked carefully disregarding the inspection results and meat examination.

Refereces

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