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Original Article

Microscopic and Molecular Detection of *Sarcocystis cruzi* (Apicomplexa: Sarcocystidae) in the Heart Muscle of Cattle

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Abstract

Background: *Sarcocystis* is a genus of coccidian protozoa that at least seven species of it can parasitize cattle. The global prevalence of *Sarcocystis* is close to 100% in adult cattle. The main aim of this study was to identify the infection rate of *Sarcocystis* spp. in heart of cattle in Tehran, Iran by microscopy and PCR-RFLP methods.

Methods: Totally, 100 bovine heart samples were collected from the main slaughterhouse of Shahriar, Meysam slaughterhouse, west of Tehran in 2016. At first, heart samples were completely examined for the presence of sarcocystic macrocysts. Then, for microscopic examination, 50 g of each heart was digested in sterile condition using pepsin acid digestion method. Then, the species of the parasite were detected by PCR-RFLP technique and sequencing.

Results: Overall, 97 of 100 of the heart muscle samples were infected with *Sarcocystis*. All the samples were detected as *S. cruzi* through similar patterns in PCR-RFLP.

Conclusion: *S. cruzi* is the most common species in the heart of cattle slaughtered in Shahriar.

Introduction

Sarcocystis is a genus of coccidian parasites that belongs to the phylum apicomplexa and usually encysts in the muscles of herbivores. This genus has more than 200 different species (1). This protozoa is an intracellular parasite and is the most common parasites of livestock (2, 3). As the cattle are common intermediate hosts of this parasite, the global prevalence of *Sarcocystis* in most regions studied, is close to 100% in

adult cattle (4). In the life cycle of this parasite human and other animals, act as intermediate and final hosts. Depending on the types of *Sarcocystis*, human can be definitive or intermediated host. Cattle are intermediate hosts for seven species of *Sarcocystis* and humans are the definitive hosts for *Sarcocystis hominis* and *Sarcocystis heydorni* (5-7).

Acute infection renders weight loss, anemia, abortion, fever, anorexia, while in chronic phase tissue cysts are formed in skeletal and cardiac muscles without any traceable symptoms. Some of the cysts are recognized during slaughterhouse inspection (macrocysts), but other types are not visible with naked eyes (microcysts). The prevalence of infection in animals varies in different regions (8, 9). The rate of infection in cattle was more than 90% in Iran (10, 11). This parasite is of great importance due to economic losses and public health issues.

The conventional method for detecting *Sarcocystis* species is based on the cyst wall structure by light and electron microscopy (3, 12, 13). For example, *S. cruzi* and *hirsuta* have thin and thick wall, respectively (6). *S. cruzi* (thin wall) easily distinguishes from *S. hominis* and *S. hirsuta* (thick wall) by light microscopy, but the morphological characterization between *S. hominis* and *hirsuta* can only be detected by electron microscopy (14). However, this method is expensive, time-consuming and less common for a large number of samples (15, 16). The histopathologic study on the new tissue samples also distinguishes thin and thick walls, but this method can't differentiate the species of thick wall cysts (*S. hominis* and *S. hirsuta*) (17, 18). In the recent years nucleic acid based methods have been used to detect and determine the genotype of *Sarcocystis* species. Molecular techniques such as PCR (Polymerase chain reaction), RAPD-PCR and PCR-RFLP (restriction fragment length polymorphism) can be used to identify and distinguish between cysts in host tissue (13, 16, 19, 20). The most favorable predilection site for chronic sarcocystosis is the heart muscle (3, 12). In addition, cardiac muscle is usually used as a food in Iran.

We aimed to identify the prevalence of *Sarcocystis* spp. in heart of cattle in Shahriar County of Tehran, Iran by microscopy and PCR-RFLP techniques.

Materials and Methods

Sampling

This study was conducted at Meysam slaughterhouse located in Shahriar County (Robat Karim road), west of Tehran, capital of Iran. Cattle being indigenous to the area are usually brought for slaughtering to this slaughterhouse; hence, we selected this slaughterhouse. In addition, all sampled animals had Iranian origin. One hundred heart muscle samples of adult cattle were collected randomly from August to November 2016. The samples were collected from male Holstein breed cattle and sampling was carried out irrespective of age characteristics.

The study was approved by the Ethical Committee of Tarbiat Modares University, Faculty of Medical Sciences.

Evaluation of macroscopic and microscopic cysts

At first, heart samples were completely evaluated for the presence of sarcocystic macrocysts. In laboratory, external surface and depth of the samples were examined for macroscopic cysts by lamina cuts. For microscopic evaluation, 50 g of each heart was digested in sterile condition as described of Dubey et al. (2016) with some modification (21). Briefly, 50 g of each sample was mixed in 100 ml of pepsin-hydrochloric acid digestion (2.5 g pepsin, 5 g Nacl, 10 ml Hcl, and 990 ml distilled water). Supernatant phase was incubated in 37 °C for 4-5 h by rotating at 120 rpm. The digested samples were passed through double layer gauze. Flow-through was centrifuged at 2000 ×g for 10 minutes and sediment suspended in 0.5 ml of distilled water. A drop of it was

checked for the bradyzoite with high magnification of light microscope (Zeiss, Axiostar plus).

Molecular identification

Amount of 25 mg of sediment solution was transferred to 1.5 ml tube and store at -20 °C until DNA extraction. DNA of the samples was extracted with DNA purification kit (YTA Genomic DNA Extraction Mini kit for Tissue, made by Favorgen, Taiwan)) according to manufacturer's instruction and was kept at -20 °C for molecular study. Later, PCR was carried out to identify *Sarcocystis* species by amplification of partial sequence of small subunit ribosomal RNA (18S rRNA) gene of *Sarcocystis*. For amplification of ~700 bp of 18s rRNA of *Sarcocystis* species, primers SarcoF 5`-CGCAAATTACCCAATCCTGA-3` and SarcoR 5`-ATTTCTCATAAGGTGCAGGAG-3`were used (15, 22). The 20 µl PCR mixture contained 1 µl of template DNA, 10 µl Taq DNA Polymerase 2x Master Mix RED (Ampliqon, 2mM MgCl2) and 1 µM of each primer. The reaction was performed for 35 cycles (95 °C for 30 s, 57°C for 30 s, and 72 °C for 1 min), with an initial denaturation (95 °C for 5 min) and a final extension (72 °C for 10 min).The positive amplicons were digested by BfaI and MnlI (Thermo Fisher Scientific) restriction enzyme. The reaction mixture was incubated at 37 °C for 4 h (5 µl PCR reaction mixture, 10 U of the restriction enzyme, 1 µL buffer and 9 µL of the Nuclease-free water). The PCR product was analyzed with 3.5% agarose gel. Table 1 shows RFLP patterns for *Sarcocystis* species by two enzymes MnlI and BfaI. For verification of RFLP pattern, four samples were selected randomly and were sent to Bioneer Company (South Korea) for nucleotide sequencing. The sequences were edited and aligned using ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and compared with reference sequences from GenBank.

Table 1 : The enzyme digestion pattern for *Sarcosystis* species by two enzymes *Mnll* and *Bfal*

Species	***MnlI***	***BfaI***
Sarcocystis hominis	370/190/90 Bp	560/70/50 Bp
Sarcocystis cruzi	370/190/90 Bp	365/195/70/50 Bp
Sarcocystis hirsuta	185/170/120/90 Bp	570/70/50 Bp
Sarcocystis heydorni	200/190/180/90 Bp	370/195/70/50 Bp
Sarcocystis rommeli	355/190/90 Bp	350/195/70/50 Bp
Sarcocystis bovifelis	355/190/90 Bp	350/195/70/50 Bp
Sarcocystis bovini	355/190/90 Bp	350/195/70/50 Bp

For *Sarcocystis* species, the phylogenetic tree was built with the maximum likelihood algorithm using molecular evolutionary genetics analysis (MEGA) software (version 7.0), including sequences of representative species of *Sarcocystis* infecting cattle from the GenBank.

Results

No cysts were observed in macroscopic observation. In the microscopic study, 97% of samples were positive for sarcocyst (Fig. 1).

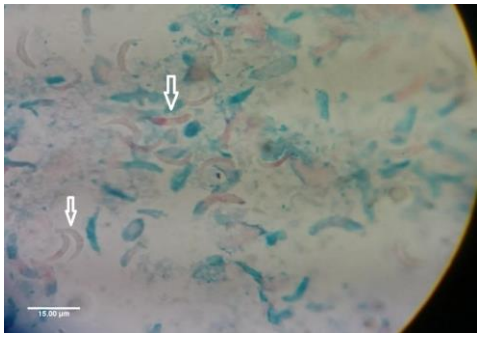

Fig. 1: *S. cruzi* bradyzoites released from a sarcocystic cyst using pepsin-hydrochloric acid digestion method and stained with Giemsa (Magnification, ×1,000)

In molecular study, out of 100 samples, 97(97%) were positive and showed a band size~700 bp in gel electrophoresis (Fig. 2).

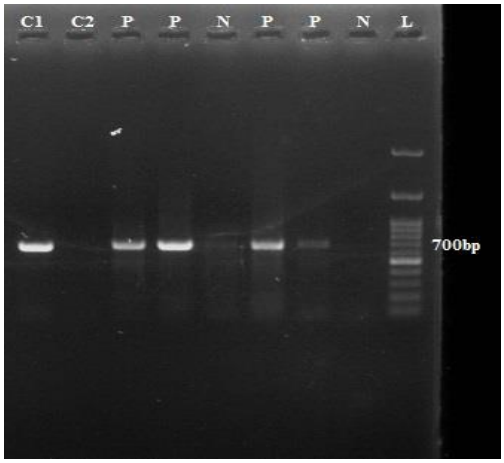

Fig. 2: PCR products of 18SrRNA gene of *Sarcocystis*. L: 100bpDNA ladder; C1: positive control; C2: negative control; P: positive sample; N: negative sample

The RFLP pattern showed that positive samples were *S. cruzi*. Fig. 3 shows the RFLP patterns for *Sarcocystis* species. The analysis of the 18s rRNA sequences confirmed the results of RFLP. Four samples that were sequenced, had 99% homology with *S. cruzi*.

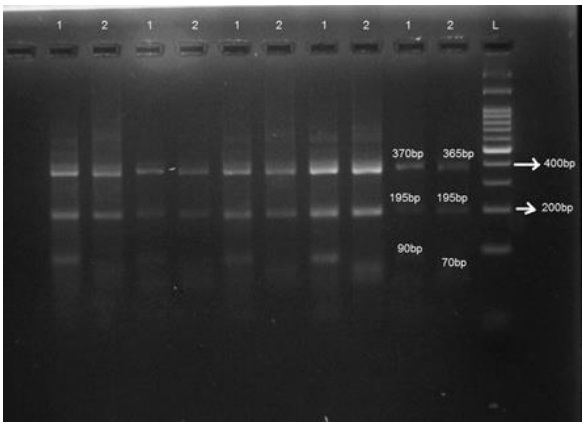

Fig. 3 : RFLP pattern of PCR products. L: 100bp DNA Ladder 1: RFLP with BfaI enzyme 2: RFLP with MnlI enzyme

In Fig. 4, the phylogenetic relationship of isolated *S. cruzi* in the current study was compared with some other species of coccidia available in the GenBank using a phylogeny tree.

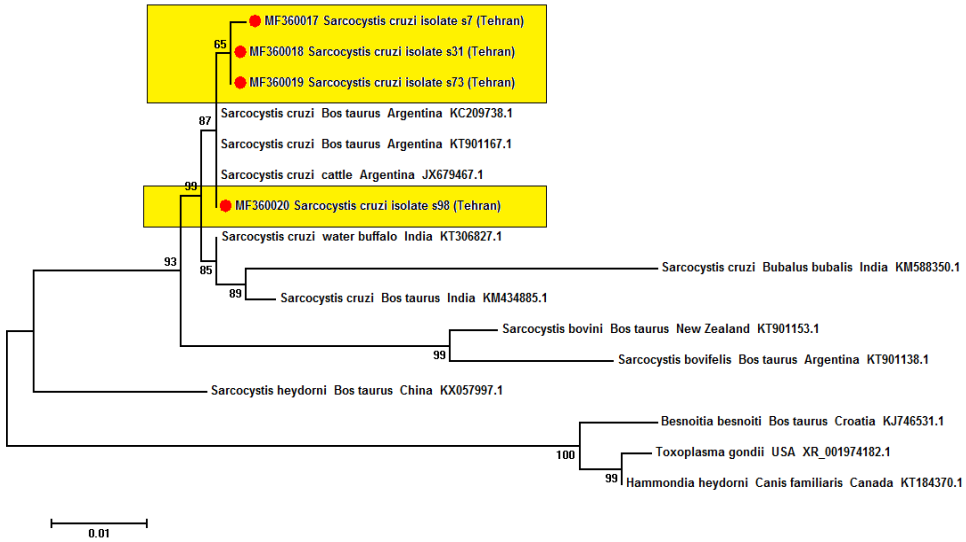

Fig. 4 : Phylogenetic relationships based on partial sequence of small subunit ribosomal RNA (18S rRNA) gene of *Sarcocystis cruzi* isolated from cattle heart muscle in this study with GenBank reference strains. Sequences from this study are highlighted with yellow color and bold accession numbers

The sequences of the isolates were deposited in GenBank under accession numbers of MF360017, MF360018, MF360019 and MF360020 (Table 2).

Table 2: The name of the species along with their accession numbers

Species	*Accession number*
[*Sarcocystis cruzi*] isolate s7	MF360017
[*Sarcocystis cruzi*] isolate s31	MF360018
[*Sarcocystis cruzi*] isolate s73	MF360019
[*Sarcocystis cruzi*] isolate s98	MF360020

Discussion

In the present study, 97% of the heart samples were found to be positive for *Sarcocystis* and were identified as *S. cruzi* (100%). These findings should be considered by the veterinary organization, because *S. cruzi,* despite having no pathologic effects on humans, has severe effects on the livestock and can lead to severe clinical sign, abortion, weight loss, neurologic sign, fever, loss of animal products and death (23, 24).

On international global scale, several investigations have reported various percentage rates for *Sarcocystis* prevalence in the heart muscle of cattle. In 90 beef cattle from Argentina, 100% of cattle were found infected with *S. cruzi* (25). In a study (26) performed on 50 heart muscle samples using technique of concentration in Brazil, *S. cruzi* was found in all of the heart muscle samples (100%). In a histopathological study (27) in Karaj (close to Tehran), 121 of 125 (96.8%) slaughtered cattle were infected with *S. cruzi* that had a thin wall cysts and 34.4% of them were infected with *S. hominis* or *hirsuta* that had a thick wall cysts. The positive rate for the thin wall cysts was 96.8% in heart, 62.4% in esophagus, 60% in skeletal muscle and 66.4% in diaphragm. In addition, the positive rate of thick wall cysts was 18.4% in diaphragm, 34.4% in esophagus and 26.6% in the skeletal muscle. The remarkable point was the absence of *S. hominis* and *S. hirsuta* in the heart muscle of cattle (27). In a study (23), 25 specimens of the diaphragm, cardiac and skeletal muscle were examined by PCR-RFLP method and all of the samples were infected to *S. cruzi*. In another study (16), 110 beef samples were examined to determine the prevalence of *Sarcocystis* species which 44 (40%) and 60 (54.5%) samples were found to be positive using light microscopy and PCR. Then 41 samples were sequenced and *S. cruzi* was detected in 41 out of 110 retail beef stored, whereas none of them was infected with *S. hominis*. Our results are in agreement with above studies on the prevalence of *Sarcocystis* species and the absence of *S. hominis* in the heart muscle. In a study (28) in Esfahan, 89% of cattle were infected with *S. cruzi* and 21% of them were infected with *S. hominis* and *S. hirsuta* (Thick wall). In Kerman, Iran (18), all of the cattle were infected by sarcocystosis. Another study (15) showed 73.1% of 380 cattle slaughtered in Argentina had thin wall (*S. cruzi*) and 23.1% of them had thick wall (*S. hominis* and *S. hirsuta*). Another survey conducted (29) in cattle of Italia, the prevalence of *S. cruzi, S. hirsuta* and *S. hominis* were 72.4%, 1.8% and 42.7%, respectively. Based on databases, reports of prevalence of *S. hominis* and *S. hirsuta* have been rare in the last decade; however recently, some studies related to the identification of *S. hirsuta* (30, 31) and *S. hominis* (32) have been published in Iran.

According to a PCR-RFLP study (10) on heart (n=24), diaphragm (n-24), esophagus (n=24), tongue (n=24) and intercostal muscle (n=24) of 120 slaughtered cattle in Yazd (central Iran). The results showed that 112 of 120 (93%) of samples were positive and the preva-

lence of *S. cruzi*, *S. hominis* and *S. hirsuta* were calculated 90%, 57.5% and 38.5% respectively. Of the 24 heart muscle samples, all of them were infected with *S. cruzi* and 9 and 4 samples were infected with *S. hominis* and *S. hirsuta* respectively (10). In Hungary with conventional PCR, from 151 heart and esophagus samples, 100 samples were positive and out of 36 samples were sequenced, 64% were *S. cruzi* and 19% were *S. hominis* (33). In northern Vietnam, 101 slaughtered cattle were examined using PCR-RFLP method and the prevalence of *S. cruzi*, *S. hominis* and *S. hirsuta* were reported to be 54.5%, 27.7% and 53.5%, respectively (34). The three above-mentioned studies showed a high prevalence of *S. cruzi* similar to our study, but it differs from our study in view of *S. hominis*.

However, for accurate characterization of *Sarcocystis* species in cattle, different muscles must be evaluated for infection. For example, in a study that was conducted in Nigeria, the esophagus and diaphragm muscle of 200 cattle were selected for examination of *Sarcocystis* species using pepsin-hydrochloric acid digestion; the prevalence of *Sarcocystis* in this study was 42.5%, that 99% of them were *S. cruzi* (thin wall) and 4% were *S. hominis* (thick wall); 75 (88.2%) and 56 (65.9%) samples had sarcocystic cyst in the esophagus and diaphragm (24). In Africa, 200 samples of the esophagus and diaphragm were collected from the cattle and were examined with histopathological and enzymatic digestion methods that histological method showed 80% of cattle infected with *Sarcocystis* species while the enzymatic showed 95%. *S. cruzi* (thin wall) in the diaphragm and esophagus were 94.2% and 100%, respectively. Sarcocysts with a thick wall were 4.4% prevalent in diaphragm and there was no sarcocyst in esophagus (35). Our study showed that *S. cruzi* is a dominant species in heart muscles of slaughtered cattle in Shahriar, near Tehran. In some studies conducted by microscopic examination of *Sarcocystis*, 100% of cattle were found infected with *Sarcocystis* (18, 36, 37). No cysts were observed in macroscopic observation that was in agreement with some other studies (18, 38). The lack of macroscopic cysts in this study could be due to the fact that these cysts probably originate from dogs (27).

One of the reasons for the high prevalence of *S. cruzi* in comparison with other species seems to be related to the fact that dogs as the final host for this species are more likely to be around the cattle than the other animals. On the other hand, in a dairy farm dogs are usually used as a guard and cattle have easier access to water and food supplies that are contaminated with dog faces in comparison to cats and human. Since no published data is available regarding the sarcocystosis in dogs in Tehran, it is suggested to investigate *Sarcocystis* infection in canids in Tehran province, which will give us a better understanding of the epidemiological aspects of sarcocystosis in this region.

Conclusion

Sarcocystis cruzi is the most common species in heart of cattle slaughtered in Shahriar slaughterhouse. Molecular methods such as PCR-RFLP can be convenient and cost-effective methods that can be used for diagnosis and detection of *Sarcocystis* species, even if the parasitic load is low.

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Conflict of interest

The authors declare that they have no conflict of interest.

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