



High Occurrence of *Sarcocystis* Cysts in Meat Produced in Yazd, Central Iran

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Abstract

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Background: Sarcocystosis is one of the most widespread parasitic diseases in cattle caused by three main species of *Sarcocystis* genus. The major aim of this study was to determine the prevalence of *Sarcocystis* spp. in meat produced in Yazd, central Iran with special reference to species identification, using Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP).

Methods: During March 2012 to May 2013, samples were randomly collected from esophagus, heart, diaphragm, intercostal muscle and tongue of 120 slaughtered cattle at Yazd, central Iran. After DNA extraction, PCR-RFLP was used for detection and identification of *Sarcocystis* spp. The statistical analysis was performed by Chi-Square test, using SPSS software (v. 16.0).

Results: The molecular analysis showed that *Sarcocystis* spp. was found in 112 of 120 (93.3%) slaughtered cattle. The prevalence of *S. cruzi*, *S. hirsuta* and *S. hominis* were 90%, 38.3% and 57.5% respectively. Among the 112 infected cattle, single and multiple (infected by more than one species) infection were seen in 45 and 67 cattle, respectively. No significant association was detected between sex, age, sample type and the prevalence of *Sarcocystis* spp. ($p>0.05$).

Conclusion: Considering its public health importance, high prevalence of *S. hominis* should be highlighted in this region. To the best of our knowledge, this study is the first of its kind to be conducted in Iran. More detailed studies are needed to describe the distribution pattern and species identification of *Sarcocystis* spp. in other regions of Iran.

Introduction

Sarcocystosis is one of the most widespread parasitic diseases in cattle caused by three main species of *Sarcocystis*

genus belonging to phylum Apicomplexa. These are *S. cruzi* (syn. *S. bovicanis*), *S. hirsuta* (syn. *S. bovis*) and *S. hominis* (syn. *S. bovis*), having two-host life cycle in which canids, felids and humans are their final hosts,

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respectively and cattle acts as the intermediate host (Bucca et al., 2011; Ghisleni et al., 2006; Hajimohammadi et al., 2014a; Moghaddam Ahmadi et al., 2014; Moré et al., 2011; Oryan et al., 2011).

The life cycle of *Sarcocystis* spp. is divided into sexual and asexual phases occurring in final and intermediate hosts, respectively. Oocysts harboring two sporocysts are expelled by feces of the final hosts. If an intermediate host such as cattle ingests the oocysts or free sporocysts, the infectious cysts (sarcocysts) develop after several evolution stages in the skeletal and cardiac muscles. The final hosts are infected by consumption the contaminated tissues of the infected animal (Abdel-Ghaffar et al., 2009; Dubey and Morales, 2006; Dubey et al., 2006; Dubey et al., 2007; Motamedi et al., 2011; Oryan et al., 1996; Valinezhad et al., 2008).

In general, the disease in cattle has no typical acute characteristics. Lower growth and weight loss result in economic damages and are the main complication of illness in the infected cattle. However, eosinophilic myositis that is a severe form of disease with considerable clinical signs may, in some instances, occur in the severely infected cattle (Bucca et al., 2011; Wouda et al., 2006). Human sarcocystosis is mainly characterized by gastrointestinal discomforts such as diarrhea, vomiting, stomachache and nausea after consumption of semi-cooked beef containing *S. hominis* (Fayer, 2004; Oryan et al., 2010; Tappe et al., 2013).

PCR-RFLP is an accurate, efficient and sensitive method to study and identify some species of parasites such as *Sarcocystis* spp. in cattle (Boughattas and Salehi, 2014; Hajimohammadi et al., 2014a; Moré et al., 2011). Most previous epidemiological investigations performed by other methods such as impression smear, digestion or histology methods on cattle sarcocystosis in the world, showed diagnostic limitation in distinguishing the *S. hominis* from the *S. hirsuta* (thick-walled cysts). Electron microscopy could be used as an accurate differential key for species identification in differentiating the morphological structures of the sarcocysts walls (Dubey et al., 1989; Jehle et al., 2009; Moghaddam Ahmadi et al., 2014; Oryan et al., 2010; Valinezhad et al., 2008). However, this method is time consuming and costly, especially when the sample size is too high.

The major aim of this study was to identify the prevalence of *Sarcocystis* spp. in meat produced in Yazd, central Iran with special reference to species identification, using PCR-RFLP.

Materials and methods

Sampling

This study was conducted at Yazd slaughterhouse. Yazd is the capital of Yazd province located in central part of Iran with dry and hot climate. The city is almost the main driest

city of the country, with an average annual rainfall of 60 mm and average percentage humidity of 31.5%. Yazd is at 1203 m above sea-level, and covers 16,000 km² (Wikipedia, 2013).

During March 2012 to May 2013, among 120 slaughtered cattle, 74 males and 46 females at Yazd slaughterhouse, samples were randomly collected from the esophagus (n=24), heart (n=24), diaphragm (n=24), intercostal muscle (n=24) and tongue (n=24). The slaughtered cattle were categorized into three age groups including <2 (n=24), 2-4 (n=74) and 4< (n=22) years old. The samples (each one about 50 g) were obtained during routine official meat inspection while naked eye observation was done to find the macroscopic sarcocysts. The samples were then immediately transferred to laboratory and stored at -20 °C for further molecular studies.

DNA extraction

Genomic DNA was extracted from 30 mg of each sample, using salting out method. The samples were crushed and then lysed with 900 µl NET buffer (NaCl, 50 mM; EDTA pH 8, 25 mM; Tris-HCl pH 7.6, 50 mM) supplied by 10 µl proteinase K (Thermo Scientific, EO049, 20 mg/ml) and Sodium Dodecyl Sulfate (SDS) with final concentration of 1%. The lysis stage was completed after an overnight incubation at 56 °C. Purification of DNA was done by adding 250 µl NaCl 6 M. After centrifugation, the supernatant was transferred to a new sterile 1.5 ml microtube and then precipitated by cold absolute ethanol. After washing with ethanol 70%, the pellet was diluted in 100 µl double distilled water and stored in -20 °C until next examination (Hajimohammadi et al., 2014b).

Molecular identification

Species of *Sarcocystis* was detected by amplification of nucleotide sequences particular 18s rRNA gene, using specific primers of SarF 5'-CGT GGT AAT TCT ATG GCT AAT ACA-3' and Sar R 5'-TTT ATG GTT AAG ACT ACG ACG GTA-3'. Based on databases, the amplicon size of *S. hominis*, *S. hirsuta* and *S. cruzi* were 926 bp, 953 bp and 937 bp, respectively (Hajimohammadi et al., 2014b).

Amplification was performed using 1X PCR buffer, 1.5 mM MgCl₂, 0.2 mM each dNTP, 1U Taq DNA polymerase, 10 pmol each primer and 100 ng genomic DNA as a template. The amplification program was done with an initial denaturation of 94 °C for 5 min, followed by 30 cycles of 94 °C for 60 s, 58 °C for 60 s and 72 °C for 60 s and finalized with extension of 72 °C for 5 min. The amplicons were analyzed on 1% agarose gel, using electrophoresis alongside of 100 bp DNA ladder (Hajimohammadi et al., 2014b).

Appropriate restriction enzymes were chosen for species detection of *S. hominis*, *S. cruzi* and *S. hirsuta* (Yang et al.,

2002). RFLP analysis was performed, using *BfaI* and *RsaI* restriction enzymes. The reaction was carried out with 10 U either *BfaI* or *RsaI* restriction enzyme, 1X specific buffer and 10 µl PCR products within an incubation of 16 h at 37 °C according to the manufacturer's recommendations. The digestion was analyzed using agarose gel electrophoresis alongside with 100 bp DNA ladder. After RFLP with *BfaI*, the restriction fragments of 376 bp and 397 bp detected *S. hominis* or *S. hirsuta* and 184 bp, 371 bp as well as 382 bp fragments detected *S. cruzi*. The restriction enzyme of *RsaI* was used in order to distinguish *S. hominis* from *S. hirsuta*. After RFLP with *RsaI*, the restriction fragments of 376 bp and 577 bp detected *S. hirsuta* and fragment 926 bp, without any digestion, detected *S. hominis*. For verification, each species detected in the samples was randomly selected and sent for sequencing and the results were analyzed with BLAST. The isolate regarding to *S. cruzi* is getting indexed in Genbank with accession number of KF933850.

Statistical analysis

The statistical analysis was performed by Chi-Square test,

using SPSS software (v. 16.0), and the $p < 0.05$ level was considered significant.

Results

The *Sarcocystis* spp. was found in 112 of 120 (93.3%) cattle slaughtered at Yazd slaughterhouse.

None of the samples were infected with macroscopic sarcocysts. The prevalence of *S. cruzi*, *S. hirsuta* and *S. hominis* were 90%, 38.3% and 57.5%, respectively (Table 1).

Among the 112 infected cattle, single and multiple (more than one species) infections were seen in 45 and 67 cattle, respectively. No significant associations were detected between sex and age of cattle and the prevalence of *Sarcocystis* spp. ($p > 0.05$).

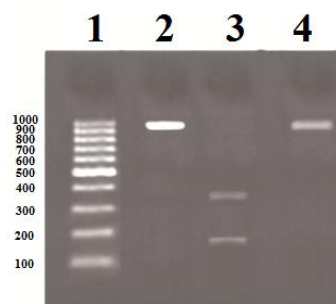
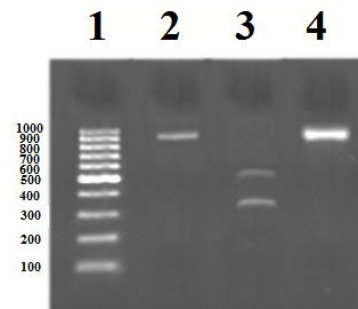
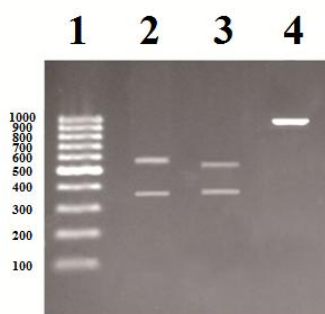
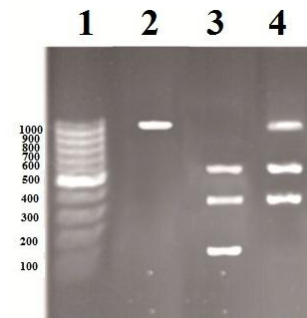
As shown in Table 2, although all the 24 samples of tongue were infected by *Sarcocystis* spp., but this prevalence was not statistically different ($p > 0.05$) with the other tissue samples including diaphragm (83.3%), heart (91.7%), esophagus (95.8%) and intercostal muscle (95.8%). Figures 1-4 demonstrate PCR-RFLP analysis of *Sarcocystis* spp.

Table 1: Prevalence of *Sarcocystis* spp. in slaughtered cattle in Yazd, Iran in different age groups

	Age groups (Year)			Total
	<2	2-4	4<	
	Number of slaughtered cattle			
	24	74	22	120
<i>Sarcocystis</i> spp.	Number of samples with parasites (%)			
<i>S. cruzi</i>	6 (25)	29 (39.2)	6 (27.3)	41 (34.2)
<i>S. hirsuta</i>	0 (0)	0 (0)	1 (4.5)	1 (0.8)
<i>S. hominis</i>	3 (12.5)	0 (0)	0 (0)	3 (2.5)
<i>S. cruzi</i> and <i>S. hirsuta</i>	1 (4.2)	0 (0)	0 (0)	1 (0.8)
<i>S. cruzi</i> and <i>S. hominis</i>	5 (20.8)	12 (16.2)	5 (22.7)	22 (18.3)
<i>S. hirsuta</i> and <i>S. hominis</i>	0 (0)	0 (0)	0 (0)	0 (0)
<i>S. cruzi</i> and <i>S. hirsuta</i> and <i>S. hominis</i>	9 (37.5)	28 (37.8)	7 (31.8)	44 (36.7)
Total	24 (100)	69 (93.2)	19 (86.4)	112 (93.3)

Table 2: Prevalence of *Sarcocystis* spp. in different tissues of slaughtered cattle in Yazd, Iran

<i>Sarcocystis</i> spp.	Tissue type (number)					Total (120)
	Diaphragm (24)	Heart (24)	Esophagus (24)	Tongue (24)	Intercostal muscle (24)	
	Number of samples with parasites (%)					
<i>S. cruzi</i>	5 (20.8)	13 (54.2)	7 (29.2)	10 (41.7)	6 (25)	41 (34.2)
<i>S. hirsuta</i>	1 (4.2)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0.8)
<i>S. hominis</i>	0 (0)	0 (0)	1 (4.2)	0 (0)	2 (8.3)	3 (2.5)
<i>S. cruzi</i> and <i>S. hirsuta</i>	0 (0)	0 (0)	1 (4.16)	0 (0)	0 (0)	1 (0.8)
<i>S. cruzi</i> and <i>S. hominis</i>	2 (8.3)	5 (20.8)	3 (12.5)	1 (4.2)	11 (45.8)	22 (18.3)
<i>S. hirsuta</i> and <i>S. hominis</i>	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
<i>S. cruzi</i> and <i>S. hirsuta</i> and <i>S. hominis</i>	12 (50)	4 (16.7)	11 (45.8)	13 (54.2)	4 (16.7)	44 (36.7)
Total	20 (83.3)	22 (91.6)	23 (95.8)	24 (100)	23 (95.8)	112 (93.3)

**Fig. 1:** PCR-RFLP analysis. Lane 1: 100 bp DNA ladder; Lane 2: PCR product of target gene; Lane 3: RFLP with *BfaI* (184 bp, 371 bp and 382 bp for *S. cruzi*) Lane 4: RFLP with *RsaI* (no digestion for *S. cruzi*)**Fig. 3:** PCR-RFLP analysis. Lane 1: 100 bp DNA ladder; Lane 2: RFLP with *RsaI* (no digestion for *S. hominis*); Lane 3: RFLP with *BfaI* (376 bp and 550 bp for *S. hominis*); Lane 4: PCR product of target gene**Fig. 2:** PCR-RFLP analysis. Lane 1: 100 bp DNA ladder; Lane 2: RFLP with *RsaI* (376 bp and 577 bp for *S. hirsuta*); Lane 3: RFLP with *BfaI* (397 bp and 557 bp for *S. hirsuta*); Lane 4: PCR product of target gene**Fig. 4:** PCR-RFLP analysis. Lane 1: 100 bp DNA ladder; Lane 2: PCR product of target gene; Lane 3: RFLP with *BfaI* (184 bp, 371 bp and 382 bp for *S. cruzi*, 376 bp and 550 bp for *S. hominis*, 397 bp and 557 bp for *S. hirsuta*) Lane 4: RFLP with *RsaI* (376 bp and 577 bp for *S. hirsuta*, no digestion for *S. cruzi* and *S. hominis*)

Discussion

This study determined the prevalence of sarcocystosis and identified the species of *Sarcocystis* spp. in cattle slaughtered in Yazd, central Iran. The previous examina-

tions in other parts of this country revealed high prevalence of cattle sarcocystosis. Nourollahi-Fard et al. (2013) showed that 121 out of 125 (96.8%) slaughtered cattle in Karaj, Iran were infected by thin-walled cysts of *S. cruzi*, while 43 out of them (34.4%) had thick-walled *Sarcocystis*

cyst (*S. hominis* or *S. hirsuta*). Nourani et al. (2010) declared that 89% of cattle in Isfahan, Iran were infected by *S. cruzi*, while thick-walled sarcocysts were detected in 21% of cattle. According to another survey performed in Kerman, eastern Iran, 100% of cattle were found infected by sarcocystosis (Nourollahi Fard et al., 2009). In all the previous surveys, impression smear, histological or digestion techniques were used in detection of *Sarcocystis* spp. Since none of the indicated assays could identify *S. hominis* and *S. hirsuta* (Jehle et al., 2009; Moré et al., 2011), the prevalence of these species in cattle population of Iran had not been reported earlier. In the present study, we applied molecular assay (PCR-RFLP) for detection of all three known species of *Sarcocystis* in cattle of central Iran.

The prevalence of *S. cruzi*, *S. hirsuta* and *S. hominis* in 101 slaughtered cattle in Northern Vietnam has been reported 54.5%, 27.7% and 53.5%, respectively (Jehle et al., 2009). *Sarcocystis* was detected in samples of 73.1% of 380 cattle slaughtered in Argentina of which 71.5% had *S. cruzi* and 23.1% contained thick walled *Sarcocystis* spp. (Moré et al., 2011). Another survey by Domenis et al. (2011) showed that infection rate of *S. cruzi*, *S. hirsuta* and *S. hominis* in cattle of Italy were 74.2%, 1.8% and 42.7%, respectively. Pritt et al. (2008) detected *S. cruzi* in 41 out of 110 retail beef stored in Vermont, USA, while no samples were infected by *S. hominis*. The prevalence of *Sarcocystis* spp. in the Brazilian and Argentinean beef was reported as 6.25% and 23.44%, respectively (Ghisleni et al., 2006). Our results are almost comparable to the study of Jehle et al. (2009) that reported 50.5% multiple infections with different *Sarcocystis* spp. in cattle.

Similar to our findings, several examinations have been carried out at different parts of the world indicating high prevalence of *S. cruzi* (Böttner et al., 1987; Bucca et al., 2011; Fukuyo et al., 2002; Ghisleni et al., 2006; Jehle et al., 2009; Latif et al., 1999; Moré et al., 2011; Obijiaku et al., 2013; Pritt et al., 2008). However, based on databases, there are few published reports about the prevalence of *S. hirsuta* and *S. hominis*. In the past, these two species of *Sarcocystis* could only be identified by electron microscopy (Domenis et al., 2011; Jehle et al., 2009; Moré et al., 2011), but recently, there have been published some documents related to the first molecular identification of *S. hirsuta* (Eslami et al., 2014) and *S. hominis* (Hajimohammadi et al., 2014b) in native cattle of Iran.

One of the most important reasons for the higher prevalence of *S. cruzi* in comparison to the other species of *Sarcocystis* in cattle seems to be related to the fact that the presence of dogs as the final host, in the vicinity of cattle is more common. On the other hand, the cattle could get access easier to feed or water infected with feces of dogs compared to the other two final hosts (cat and human). However, the relatively high prevalence of *S. hominis* in

cattle of central Iran is surprising and also warning. Because, it shows that the human's wastewater is not properly managed and the cattle are in contact to the human fecal sources. This threatening prevalence in Yazd area could possibly be due to infection of water sources to the human feces (Fayer, 2004; Xiang et al., 2009). As, Yazd is a dry city and water shortage is common in this area, non-hygienic water sources may be used for agriculture and animal husbandry. Therefore, there is a serious risk that the people of this region get infected by *S. hominis*. Since no published data are available regarding the intestinal sarcocystosis in Iranian people, the fecal sample of those human populations who are at high risk should be tested for detection of *S. hominis* sporocysts/oocysts (Xiang et al., 2009).

Absence of the macroscopic forms of sarcocysts in this study is in agreement with most of the previous investigations which stated that this issue is related to lesser association of cattle with cat compared to the other final hosts (Nourollahi Fard et al., 2009; Nourollahi-Fard et al., 2013; Obijiaku et al., 2013). On the other hand, development of macrocysts of *S. hirsuta*, lasts up to several years, while cattle are usually slaughtered earlier (Nourani et al., 2010).

As seen in Table 2, while other forms of multiple infections were prevalent, a characteristic noticeable and somewhat confusing finding, in the present study, it was lack of joint infection of *S. hirsuta* and *S. hominis*. However, more detailed investigations regarding detection of infection in the final hosts of Yazd province including humans, stray cat and dog could result in achieving informative data in order to clarify this issue.

This study showed high prevalence of *Sarcocystis* in all tested tissue samples including tongue, diaphragm, heart, esophagus and intercostal muscle. This finding is in accordance with most other studies showing all of these tissues as the predilection sites of *Sarcocystis* spp. (Fayer, 2004; Fukuyo et al., 2002; Jehle et al., 2009; Nourani et al., 2010; Nourollahi Fard et al., 2009; Oryan et al., 1996; Oryan et al., 2010). In this study, no significant association was found between sex, age and the prevalence of *Sarcocystis* spp. that is in agreement with most of the previous investigations (Jehle et al., 2009; Nourollahi-Fard et al., 2013; Obijiaku et al. 2013).

Since sarcocystosis is often asymptomatic, there is no practical drug-therapy for treatment of the infected cattle. Therefore, emphasis on the preventive actions is a necessary issue in reducing the infection. Principal stage to reach this purpose is the training of farmers about *Sarcocystis* life cycle and its zoonotic feature. Considering its public health importance, high prevalence of *S. hominis* should be highlighted in this region. The people who consume semi-cooked barbecued beef (Kebab) should be warned. Although the eosinophilic myositis resulting from the cattle sarcocystosis is not prevalent (Wouda et al., 2006), it

seems education of the meat inspectors regarding the signs of this lesion in carcasses is an effective effort. Keeping beef at -20 °C for 24 h or -4 °C for 48 h and heating them to a core temperature of 70 °C make it safe to eat (Fayer, 2004; Ghisleni et al., 2006).

Conclusion

To the best of our knowledge, this study is the first of its kind to be conducted in Iran indicating high prevalence of all species of cattle *Sarcocystis*. More detailed studies are needed to describe the distribution pattern and species identification of *Sarcocystis* spp. in other regions of Iran.

Conflicts of interest

The authors declare no conflicts of interest.

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