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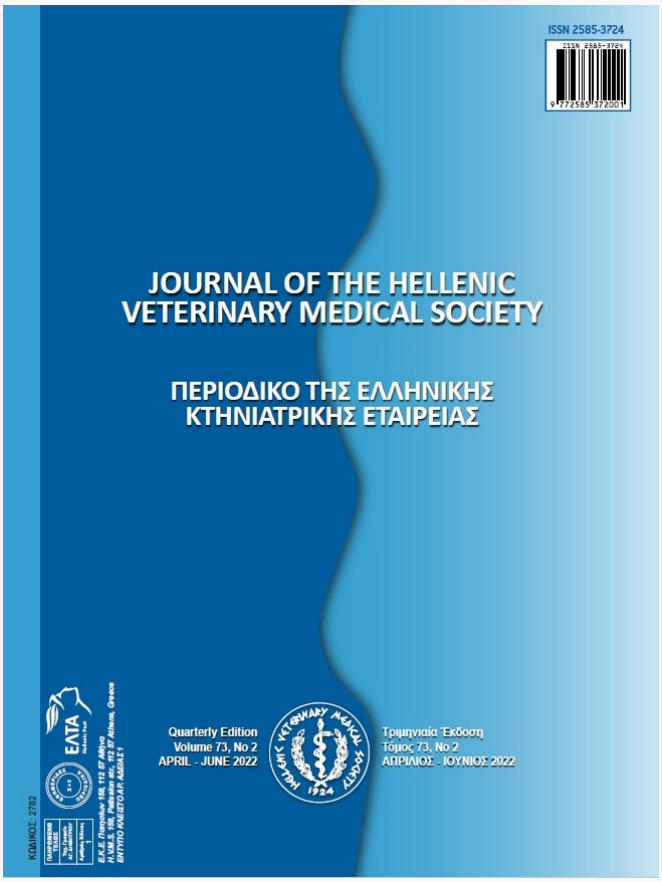

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Research article
Ερευνητικό άρθρο

Sarcocystis sinensis in slaughtered cattle from Central of Iran

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ABSTRACT: *Sarcocystis* is a food borne parasite as an intracellular parasite with two-host life cycle. Humans serve as either intermediate or definitive hosts among different species. Sarcocystosis is a very important disease because of its widespread prevalence with an impact on production and animal health. We aimed to assess *S. sinensis* in cattle from the desert area of Iran, using conventional PCR, targeting 18S rRNA. Also, the phylogenic analysis was done to mention isolate and the related ones in Genbank.

From the 108 beef samples of heart, esophagus, intercostal muscle, diaphragm, and tongue harboring *Sarcocystis* spp. stored in the BioBank of the Research Center for Food Hygiene and Safety, Shahid Sadoughi University of Medical Sciences, Yazd, Iran, DNA extraction was done. Amplification was done using the primer pair of SAR-F: 5'-TGGCTA-ATACATGCGCAAATA-3' and SAR-R: 5'-AACTTGAATGATCTATCGCCA-3'

For the target of 18S rRNA gene sequencing was done. The interesting sequence was edited and then analyzed with BLAST. Then, multiple alignments were conducted using T-coffee. The mentioned sequence was submitted to Gen-Bank in NCBI. Evolutionary analyses were conducted in MEGA7.In order to statistical analysis of the presence of *Sarcocystis sinensis* significantly with sex, age, and location, the Fisher's Exact test was done using SPSS 16.0 (SPSS Inc. Chicago, USA).The p-value < 0.05 was considered significant.

Out of 108 samples, five isolates were identified as *S. sinensis.* The distribution of *S. sinensis* was related to age (p<0.05) but had no significant difference with gender (p>0.05). Phylogenic analysis showed a similarity of *S. sinensis* with *S. hominis*. From a food safety viewpoint, this study employed a molecular approach to identify *S. sinensis*in in cattle meat. To the best of our knowledge, this is the first report of *S. sinensis* in the desert area from Iran.

Keywords: *Sarcocystis*; Cattle; Food Safety

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INTRODUCTION

Multiple genera in apicomplexa are considered as pathogens, such as *Sarcocystis* which isan intracellular parasitewith a two-host life cycle. Humans serve as either intermediate or definitive hosts among different species (Dubey et al., 1989; Regensburger et al., 2015). More than 200 *Sarcocystis* species have been identified with wildlife, so far. However, *S. hominis* and *S. suihominis,* with respectively cattle and swine as their intermediate hosts, are the most distinguished species with humans as their definitive host.

Sarcocystosis is a very important disease because of its widespread prevalence with an impact on production and animal health. It may result in reduced milk yield, weight gain, fever, anorexia, anemia, muscle weakness, poor feeding efficiency, abortion, and death in the intermediate host. However, the symptoms are limited to nausea, vomiting, and diarrhoea in humans as definitive hosts (Bunyaratvej et al., 1992; Zuo, 1992). In the case of human beings as hosts, many other species were reported which were then determined based either on their hosts or on their cell wall structure (Dubey et al., 1989; Heckeroth and Tenter, 2007). Both of these methods are inappropriate because, in the case of the earlier method, more than one species could have the same host in their cycles, the latter one also is not so useful due to changes in the wall structure during the various stages (Fayer, 2004).

Nowadays, molecular identification is considered the best approach for the characterization of species (Stojecki et al., 2012; Gjerde, 2013). One of the most important target genes used for molecular characterization is the small-subunit (SSU) rRNA (Huong and Uggla, 1999; Li et al., 2002; Yang et al., 2001a, 2001b). Molecular approaches could be useful for the differentiation of the species. We have reported the first molecular identification of *S. hominis* from Iran using PCR-RFLP (Hajimohammadi et al., 2014c). In another study, we reported the prevalence of 57.5% for *S. hominis* in cattle meat in Yazd, Iran (Hajimohammadi et al., 2014d). Also, Hajimohammadi et al (2014a) and Moghaddam-Ahmadi et al., (2015) reported the first *S. hominis* and *S. hirsuta* from beef hamburger. However, there is still no data on the presence of *S. sinensis* in beef samples produced in this country. Therefore, the current study aimed to assess *S. sinensis* in cattle from the desert area of Iran, using conventional PCR, targeting 18S rRNA. Also, phylogenic analysis was done for mention isolate and the related ones in Genbank.

MATERIALS AND METHODS

Ethics approval and consent to participate

The ethical consideration was approved by Ethical Committee from Shahid Sadoughi University of Medical Sciences, Yazd, Iran.

Study area

One of the important provinces in the central of Iran is Yazd. Yazd province with the geographical character of 32.1006°N, 54.4342°E is inside the Dasht-e Kavir desert. This region has a rain of less than 100 mm per year with temperatures of above 40 °C in summer. This province has two slaughterhouses that many kinds of livestock are slaughtered there, including cattle, sheep, goat, and camel. In comparison with the other province in Iran, Yazd province has low slaughtering of cattle with 6685 cases in 2017 (Statistical Center of Iran, 2017).

Sampling

Based on the study of Hajimohammadi et al., (2014d), the 108 beef samples harboring *Sarcocystis* spp. had stored in the BioBank of the Research Center for Food Hygiene and Safety, Shahid Sadoughi University of Medical Sciences, Yazd, Iran. The samples were obtained from different tissues, including heart, esophagus, intercostal muscle, diaphragm, and tongue. About 5 g of each sample was isolated and inserted in a sterile 1.5 ml microtube. The demographic data was present for each beef, including age and sex.

DNA extraction

Each sample (5 g) was minced and homogenized separately in each sterile bag. The genomic DNA was extracted using modified salting-out method as described previously by Hajimohammadi et al., (2014b). The quality and quantity of the extracted DNA was analyzed using agarose gel electrophoresis (0.8%) using electrophoresis apparatus (Akhtarian, Tehran, Iran) and spectrophotometer using NanoDrop device (Thermo Fisher Scientifc, Massachusetts, USA), respectively. Also, for amplifiable verification of the extracted DNA, the amplification was done using the cattle specific primers of Cytb-F 5'-CTGCCTA-ATCCTACAAATCCTC-3' and Cytb-R 5'-CGTA-ATATAAGCCTCGTCCTAC-3' targeting *cytochrome b* gene, giving an amplicon of 197 bp (Eslami et al., 2014). The results of the amplification were assessed

using agarose gel electrophoresis (Akhtarian, Tehran, Iran) alongside with the 50 bp DNA ladder. The positive and negative control was considered in each run. All the tests were repeated in triplicate.

Detection and identification using 18SrRNA

In this study, all samples were assessed for *Sarcocystis* targeting the 18S rRNA gene using the primer pair of SAR-F: 5'-TGGCTAATACATGCGCAAATA-3' and SAR-R: 5'-AACTTGAATGATCTATCGCCA-3' (37). Amplification was performed using conventional PCR with an initial denaturation of 94 ºC for 5 min, then 40 cycles of denaturation at 94 ºC for 30 s, annealing at 63 ºC for 40 s (for 18SrRNA) and at 50 ºC for 60 s (for *cox1*) , and elongation at 72 ºC for 45 s. The final elongation at 72 ºC was performed for 5 min. The reaction solution was done in a total volume of 20 µl, containing 1X PCR buffer, 0.5µM each primer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, and 100 ng genomic DNA. Amplicon analysis was done using 3% agarose gel electrophoresis alongside with 50 bp DNA ladder.

Sequencing

For final verification and identification of DNA samples (not applicable) in fragment analysis method, the Sanger sequencing method was performed. For this, the amplicons were purified using High Pure PCR Product Purification Kit (Roche, Mannheim, Germany) and then sent for sequencing (Bioneer Company, ABI 3730XL DNA Analyzer, Korea). The interesting sequence was edited and then analyzed with BLAST (38). Then, a multiple alignments were conducted using T-coffee (39). The mentioned sequence was submitted to GenBank in NCBI.

Phylogenic analysis

Evolutionary analyses were conducted in MEGA7. The evolutionary history was inferred using the UPGMA method. The bootstrap consensus tree inferred from 500 replicates was taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) was shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method and were in the units of the number of base substitutions per site. All ambiguous positions were removed for each sequence pair.

Statistical analysis

In order to statistical analysis of the presence of *Sarcocystis sinensis* significantly with sex, age, and location, the Fisher's Exact test was done using SPSS 16.0 (SPSS Inc. Chicago,USA).The p-value < 0.05 was considered significant.

RESULTS

Analysis of the extracted DNA

The quantification assessing of the extracted DNA showed the mean concentration of 428±38 ng/µl and a mean purification of 1.73±0.26 (260/280 ratio). The qualification analysis of the extracted DNA showed a single high molecular weight band without any fragmentation (not shown). The amplifiable assessing with the specific primer pair for the cattle *cytochrome b* gene was verified by amplification of a fragment with a length of 197 bp in all 120 samples (Figure 1).

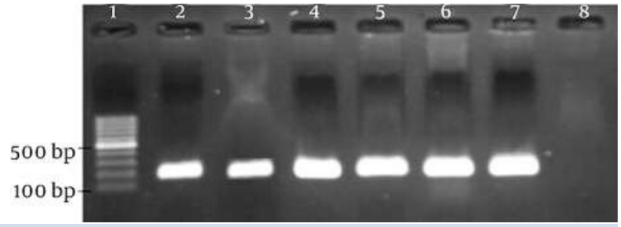

Figure 1. Agarose gel electrophoresis. Analysis for ensuring of extracted DNA for amplification. Lane 1: 100bp DNA ladder; lanes 2-7: amplicon resulted from the specific primer for cattle species identification; lane 8: negative control

Identification of *Sarcocystis* spp.

Out of 108 samples, five isolates had a band with the length of 170 bp. One of them was sequenced and after BALST analysis, it showed similarity with *S. sinensis.* The multiple sequence analysis showed our studied sequence had little nucleotide changes with the same region in some other *S. sinensis* with accession numbers of KF954728, KF954730, JX679469, KF954729, and JX679466 (Figure 2). The sequence was submitted in GenBank, NCBI with the accession numbers of KU240551. The demographic information such as age, sex, and the target organs in samples with *S. sinensis* are shown in Tables 1 to 3.

Phylogenic analysis

The phylogenic tree (Figure 3) showed that our studied sequence related to *S. sinensis* with accession number of KU240551 was more similar to the ones from *S. hominis* (AH006016 and AF006470) with difference of 0.017 using pairwise distances

```
Isolate      TTATTAGATACAGAACCAACACGCTCCATTTTTGGGGTGTAAAAGGAGGTGATTCATAGTAACCGAACG
KF954728.1   TTATTAGATACAGAACCAACACGCTCCATTCTTGGGGTGTAAAAGGAGGTGATTCATAGTAACCGAACG
KF954730.1   TTATTAGATACAGAACCAACACGCTCCATTTTTGGGGTGTAAAAGGAGGTGATTCATAGTAACCGAACG
JX679469.1   TTATTAGATACAGAACCAACACGCTCCATTTTTGGGGTGTAAAAGGAGGTGATTCATAGTAACCGAACG
KF954729.1   TTATTAGATACAGAACCAACACGCTCCATTCTTGGGGTGTAAAAGGAGGTGATTCATAGTAACCGAACG
JX679466.1   TTATTAGATACAGAACCAACACGCTCCATTTTTGGGGTGTAAAAGGAGGTGATTCATAGTAACTGAACG

cons         ****************************** ******************************** *****


Isolate      GATCGCATTATGATCATATTATTATGGTTGGCGATAGATCATTCAAGTT
KF954728.1   GATCGCATTATGATCATATTATTATGGTTGGCGATAGATCATTCAAGTT
KF954730.1   GATCGCATTATGGTCATATTATTATGATTGGCGATAGATCATTCAAGTT
JX679469.1   GATCGCATTATGGTCATATTATTATGATTGGCGATAGATCATTCAAGTT
KF954729.1   GATCGCATTATGGTCATATTATTATGATTGGCGATAGATCATTCAAGTT
JX679466.1   GATCGCATTATGGTCATATTATTATGATTGGCGATAGATCATTCAAGTT

cons         *********** ************ **********************
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Figure 2. Multiple alignments of one of the isolate with *S. sinensis* with the accession number of KF954728, KF954730, JX679469, KF954729, JX679466

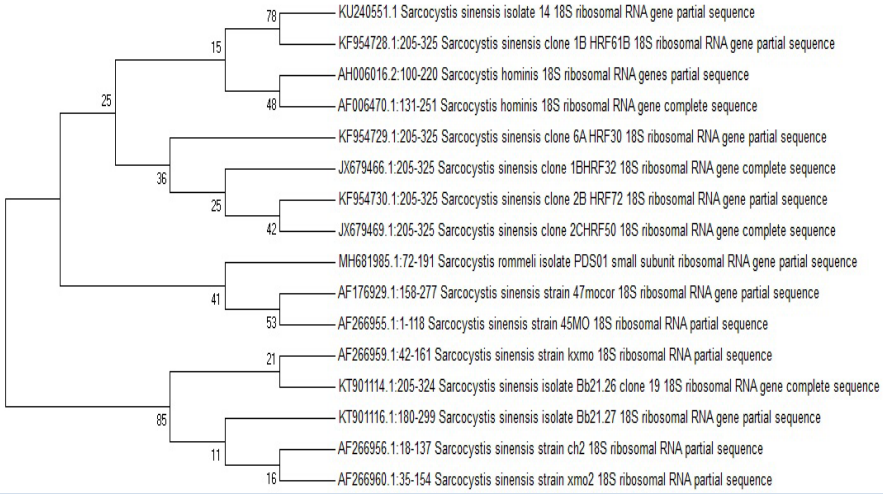

Figure 3. Phylogenic analysis of the studied isolate with the accession number of KU240551 and the other ones deposited in GenBank, NCBI, including KF954728.1: *Sarcocystis sinensis* clone 1B HRF61B 18S ribosomal RNA gene partial sequence, AH006016.2: *Sarcocystis hominis* 18S ribosomal RNA genes partial sequence, AF006470.1: *Sarcocystis hominis* 18S ribosomal RNA gene complete sequence, KF954729.1: *Sarcocystis sinensis* clone 6A HRF30 18S ribosomal RNA gene partial sequence, JX679466.1: *Sarcocystis sinensis* clone 1BHRF32 18S ribosomal RNA gene complete sequence, KF954730.1: *Sarcocystis sinensis* clone 2B HRF72 18S ribosomal RNA gene partial sequence, JX679469.1: *Sarcocystis sinensis* clone 2CHRF50 18S ribosomal RNA gene complete sequence, MH681985.1: *Sarcocystis rommeli* isolate PDS01 small subunit ribosomal RNA gene partial sequence, AF176929.1: *Sarcocystis sinensis* strain 47mocor 18S ribosomal RNA gene partial sequence, AF266955.1: *Sarcocystis sinensis* strain 45MO 18S ribosomal RNA partial sequence, AF266959.1: *Sarcocystis sinensis* strain kxmo 18S ribosomal RNA partial sequence, KT901114.1: *Sarcocystis sinensis* isolate Bb21.26 clone 19 18S ribosomal RNA gene complete sequence, KT901116.1: *Sarcocystis sinensis* isolate Bb21.27 18S ribosomal RNA gene partial sequence, AF266956.1: *Sarcocystis sinensis* strain ch2 18S ribosomal RNA partial sequence, AF266960.1: *Sarcocystis sinensis* strain xmo2 18S ribosomal RNA partial sequence

Table 1: The rate of *Sarcocystis sinensis* in slaughtered cattle in Yazd, Iran in different age groups

Age groups (Year)			
>2	2-4	4<	Total
Number of slaughtered cattle			
25	75	22	122
Number of samples with *Sarcocystis sinensis* (%)			
0 (0%)	1 (0.82)	4 (3.28)	5 (4.1)

Table 2: The rate of *Sarcocystis sinensis* in slaughtered cattle in Yazd, Iran in different sex groups

Male	Female	Total
Number of slaughtered cattle		
75	47	122
Number of samples with *Sarcocystis sinensis* (%)		
3 (2.46)	2 (1.64)	5 (4.1)

Table 3: The rate of *Sarcocystis sinensis* in different tissues of slaughtered cattle in Yazd, Iran

Diaphragm (25)	Heart (24)	Esophagus (24)	Tongue (24)	Intercostal muscle (25)	Total (122)
Number of samples with parasites (%)					
2 (1.64)	0 (0)	0 (0)	0 (0)	3 (2.46)	5 (4.1)

matrix. This analysis showed 10 groups, including Group 1: KU240551.1 and KF954728.1, Group 2: AH006016.2 and AF006470.1, Group 3: KF954729.1, Group 4:JX679466.1, Group 5: KF954730.1 and JX679469.1, Group 6: MH681985.1, Group 7: AF176929.1 and AF266955.1, Group 8: AF266959.1 and KT901114.1, Group 9: KT901116.1, and Group 10: AF266956.1 and AF266960.1.

DISCUSSION

In this study, we assessed *S.sinensi* in 108 samples from different organs of slaughtered cattle from the slaughterhouse of Yazd, Iran as in important province in the central of Iran using two different methods. Various studies have been reported different *Sarcocystis* species worldwide (Hornok et al., 2015; Domenis et al., 2011; Moré et al., 2008) and also in Iran (Hooshyar et al., 2017; Shahraki et al., 2018; Mirzaei and Rezaei, 2016). In this study, we identified *S. sinensis* in cattle for the first time in the central of Iran using the primer pair of Vangeel et al. (Farhang-Pajuh et al., 2014). In our previous study, three common species of *Sarcocystis* comprising *S. cruzi*, *S. hirsuta*, and *S. hominis* were identified with the rates of 90.0%, 38.3%, and 57.5%, respectively (Hajimohammadi et al., 2014c). In this study, we analyzed the presence of *S. sinensis* in five samples. One of them was sequenced and deposited in GenBank.

It was also found that *S. sinensis* infectionwas significantly associated with cattle for more than 4 years old (Table 1). This increasing distribution of sarcocystosis related with age has been reported in other studies (Farhang-Pajuh et al., 2014; Oryan et al., 1996). This association can be interpreted as greater age may probably cause more chances of infection, similar to what we observed for other species, such as *S. cruzi* and *S. hirsuta,* in our previous study (Hajimohammadi et al., 2014d). This finding was not in agreement with the results reported by Obijiaku et al., (2013), who found no significant difference between age and infection of sarcocystosis. The rate of *S. sinensis* between both sexes was almost equal (Table 2) and no significant difference was observed between gender and infection. This confirmed the results achieved by Obijiaku et al., (2013), but disagreed with the study of Savini et al (Savini et al., 1992), who reported the highest infection in males. However, the low number of samples infected with *S. sinensis* may effect on finding any relationship. Among different tissues, intercostal muscle and diaphragm were the only tissues positive for *S. sinensis* (Table 3).

Based on our knowledge, this is the first report of *S. sinensis* from Iran. *S. sinensis* has been reported in European countries such as Germany, Austria and Hungary, and in other parts of the world such as China and Argentina with variable prevalence (Gjerde, 2013; Yang et al., 2001b; Hornok et al., 2015; Moréet al., 2014; Dubey et al., 2015; Moré et al., 2013). Hornok et al., (2015) reported six *S. sinensis* among 82 cattle meat in Hungary. Moreover, scientists in Germany reported a divergent *Sarcocystis* taxon based on 18S rRNA similarity with *S. sinensis*, which was named *S. sinensi*-like (Moré et al., 2014; Fischer andOdening, 1998) since *S. sinensi* was considered to be species-specific for buffalo. Actually, Gjerde and Hilali (Gjerde and Hilali, 2016) also found *S. sinensis* in cattle meat and reported 95% identity with *S. hominis* at the 18S rRNA gene. In phylogenic analysis from this study, we also found more similarity of *S. sinensis* with *S. hominis.* In another study, the isolatesobtained from water buffalo in India and China were identified as *S. sinensis* using 18S rRNA gene marker (Yang et al., 2001b). Dubey et al (Dubey et al., 2015) suggested that species similar to *S. sinensis* in cattle meat should be under name of *S. rommeli* and only related species in water buffalo should be named as *S. sinensis*. In addition, Dubey et al (Dubey et al., 2016) reported that the isolates obtained from cattle named *S. rommeli*, have 99-100% homology with 18S rRNAfrom *S. sinensis* in water buffalo.

In humans, there was no clear evidence of infection by *S. sinensis* from cattle (Dubey et al., 2015). However, Chen et al (Chen et al., 2011) showed that human volunteers that ingested raw buffalo meat suffered from mild symptoms. Chen et al (Chen et al., 2011) concluded that *S. sinensis* from did not use human as a definitive host but it could represent a cause of food borne illness. Based on our knowledge, it is the first report of *S. sinensis* from the desert area of Iran, although, Hamidinejat et al (Hamidinejat et al., 2010) reported *S. sinensis* in another site of Iran, Ahvaz, West of Iran.

CONCLUSION

This study employed a molecular approach to identify *S.sinensis* in Iranian cattle meat. To the best of our knowledge, this is the first report of *S. sinensis* in Iran. Further researches are needed to achieve comprehensive data about inactivation methods of this food borne parasite in the food chain.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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