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Original Article

RFLP Analysis of Fragments of the *18S rRNA* and *Cox1* Genes to Identify *Sarcocystis cruzi* in Water Buffalo (*Bubalus bubalis*) in Guilan Province, North of Iran

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Abstract

Background: Sarcocystosis is a zoonotic disease worldwide caused by *Sarcocystis* spp., some of these species can show clinical and subclinical manifestations, resulting in financial losses. Our study was performed for identifying *Sarcocystis* sp., in slaughtered buffalo by *PCR–RFLP based* strategy with sequencing in Guilan, North of Iran.

Methods: Overall, 400 fresh muscle samples were prepared via naked-eye observation from 100 buffaloes (esophagus, diaphragm, shoulder, and thigh), followed by the digestion of samples. The PCR was done to amplify partial parts of the *18S rRNA* and mitochondrial cytochrome c oxidase subunit I (*Cox1*) genes. Then, the PCR products were digested by endonuclease SspI, DraI, and FokI. Sequencing of all species was done to confirm the RFLP results.

Results: Five macroscopic cysts (1.25%) were visible in the sample by naked-eye examination. Furthermore, 293 samples (73.25%) were found to be *Sarcocystis* sp. positive through tissue digestion and microscopic observation, whereas 376 samples (94%) were positive by PCR. In addition, the findings of PCR-RFLP and nucleotide sequence samples exhibited the infection of buffaloes with *S. cruzi*.

Conclusion: Based on the data presented herein, *Bovine sarcocystosis* caused by *S. cruzi* is very common in buffalo in the Guilan region. Regarding the high prevalence of sarcocystosis, developing disease control and prevention policies for buffaloes is necessary, and a change of attitude in *traditional farming* is recommended.

Introduction

More than 200 known species of *Sarcocystis* worldwide are capable of infecting animals (1). *Sarcocystis* is one of the most common cyst-forming species of protozoan parasites with universal distribution in different types of hosts as definitive (carnivores) and intermediate hosts (herbivores) (2-5). Some species of *Sarcocystis* may contribute to clinical symptoms, leading to weight loss, anorexia, fever, diarrhea, anemia, weakness, and death (6). Consumption of undercooked or raw beef is an important risk factor for humans (7).

Human intestinal sarcocystosis is a zoonotic disease, caused by two species of *S. fusiformis* (by consumption of infected beef) and *S. meischeriana* (by consumption of raw infected pork), resulting in digestive disorders (8). In addition to economic losses in animal husbandry, *Sarcocystis* infection can be a threat to public health (9). This parasite has a global spread and can infect various species of domestic animals. There have been many validated species in buffaloes such as *S. fusiformis,* and *S. buffalonesis* (cats as definitive hosts), as well as *S. levinei* (canids are the definitive host), and *S. dubeyii* with an unknown definitive host (10) .

Adult *Sarcocystis* of different species is distinguishable via distinct phenotypic characteristics, e.g., the shape, size, cyst wall thickness, etc. Nevertheless, these characteristics may be influenced by the location and stages of cyst, growth, parasite stage and host tissue type. Therefore, molecular methods are considered reliable for affirming species identification and differentiation (11). The variable regions of the *18S rRNA* gene are a valuable target for identifying different species (12). Molecular markers including *18S rRNA*, *28S rRNA, ITS,* and *Cox1* sequence have been effectively used to identify *Sarcocystis* (13). However, the *Cox1* is capable of differentiating *Sarcocystis* spp. thus described to be a favorable marker when compared with *18S rRNA* gene (14).

Protozoan parasites are common in Iranian domestic livestock (15). However, few studies are available in Iran for *Sarcocystis* spp. in water buffaloes (2).

We aimed at evaluating the phylogenetic and molecular data of *18S rRNA* and *Cox1* genes of *Sarcocystis* sp. in Guilan province, North of Iran.

Materials and Methods

Ethical considerations

This study was done on slaughtered buffaloes. Research Ethics committees of Islamic Azad University-Science and Research Branch approved the study with ID IR.IAU.SRB.REC.1401.126.

Sampling

From January to May 2022, esophagus, diaphragm, shoulder, and thigh muscle samples of 100 buffalo carcasses from different slaughterhouses were collected in Guilan Province, northern Iran. Fifty grams of each muscle tissue was taken by veterinarians during postmortem inspections, transported to the laboratory through a cold chain, and then stored at -20°C until further evaluation.

Trypsin digestion

Briefly, 20 g of buffalo muscles were digested in 50 ml trypsin based on the optimized protocols for 16 h at 37 °C and then centrifuged for 5 min at 7000 rpm, followed by suspending the pellet in 5 ml PBS and centrifugation for 3 min at 5000 rpm (16, 17). The pellet was suspended in 5 ml of PBS and the resulting suspension was finally applied for DNA extraction.

DNA extraction and molecular detection of Sarcocystis spp

DNA extraction was performed using a commercial DNA extraction Kit (MBST, IRAN), according to the Kit instruction (18). Amplification of *18S rRNA* and *Cox1* genes were done by primers of the *18S rRNA* gene (f: 5-TCAGGGAGGTAGTGACAAGA-3; R: 5-ATGTCTGG ACCTGGTGAGTT-3) and *Cox1* gene (f: 5-CTTTAGCGTTGTTGGTAC-3; R: 5-CCCGTAGGAATGGCAAT-3). The primers applied for *18S rRNA* (JQ713824), and *Cox1* genes (KU247899) were designed based on published sequences, and synthesized by Sinaclon Biotech Co, Tehran, Iran. PCR was performed by the standard method, except for denaturation temperature (94°C). Each 25 µl PCR reaction consisted of 0.5 µM of each primer, 0.2 mM of each dNTP, 0.2 u/µl Dream Taq buffer, 1 µg DNA template, and distilled water. The cycling conditions consisted of initial denaturation at 95 °C for 5 min, followed by 40 cycles of 94 °C for 45 s, 56 °C for 45 s and 72 °C for 45 s, and a final extension cycle at 72 °C for 10 min. Negative control of the H_2o was used as a sample. PCR products were finally electrophoresed on a 1.5% (w/v) agarose gel for 90 minutes (19).

Sequencing and phylogenetic analysis

The amplified *18S rRNA* and *Cox1* genes of *Sarcocystis* were sequenced based on a specific primer set to achieve species identification. The obtained sequences were blasted, and ClastalW V.2 software was applied to align the *18S rRNA* and *Cox1* genes sequences (20). MEGA 6.06 software was applied to generate a phylogenetic tree using Neighbor-Jinning (NJ) phylograms (with 1000 replicates) of both gene sequences (21, 22).

Results

RFLP-PCR

PCR amplification of *18S rRNA* and *Cox1* genes from of *Sarcocystis* species produced DNA fragments with the expected size of about 860 and 950 bps, respectively (Fig.1 and 2). Interestingly, RFLP analysis of PCR products with endonucleases exhibited the pattern of *S. cruzi*. As shown in Fig. 1, digestion with Dra1 and Fok1 produced 790 and 600 bps fragments of *S. cruzi*, respectively. The endonuclease Ssp1 did not result in any digestion band pattern of the *18S rRNA* gene.

In the mitochondrial gene *Cox1*, Digestion with Fok1 produced a DNA fragment of 910 bp, while the endonuclease Ssp1 and Dra1 did not result in any digestion band pattern of *Cox1*, representing *S. cruzi* (Fig. 2).

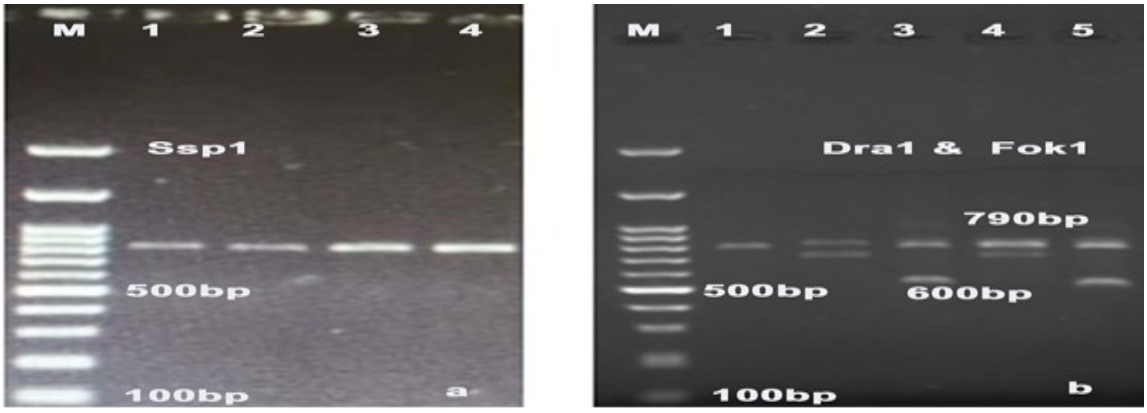

Fig. 1: PCR-RFLP analysis of 18S rRNA gene of *Sarcocystis* isolated from Iranian water buffalo demonstrating species-specific fragments of *S. cruzi*: Lane 2, 3 and 4 digested whit Ssp1(a), Lane 2 and 4 digested whit Dra1, Lane 3 and 5 digested whit Fok1(b), M marker of 100 bp, lane1(a and b) uncut (control)

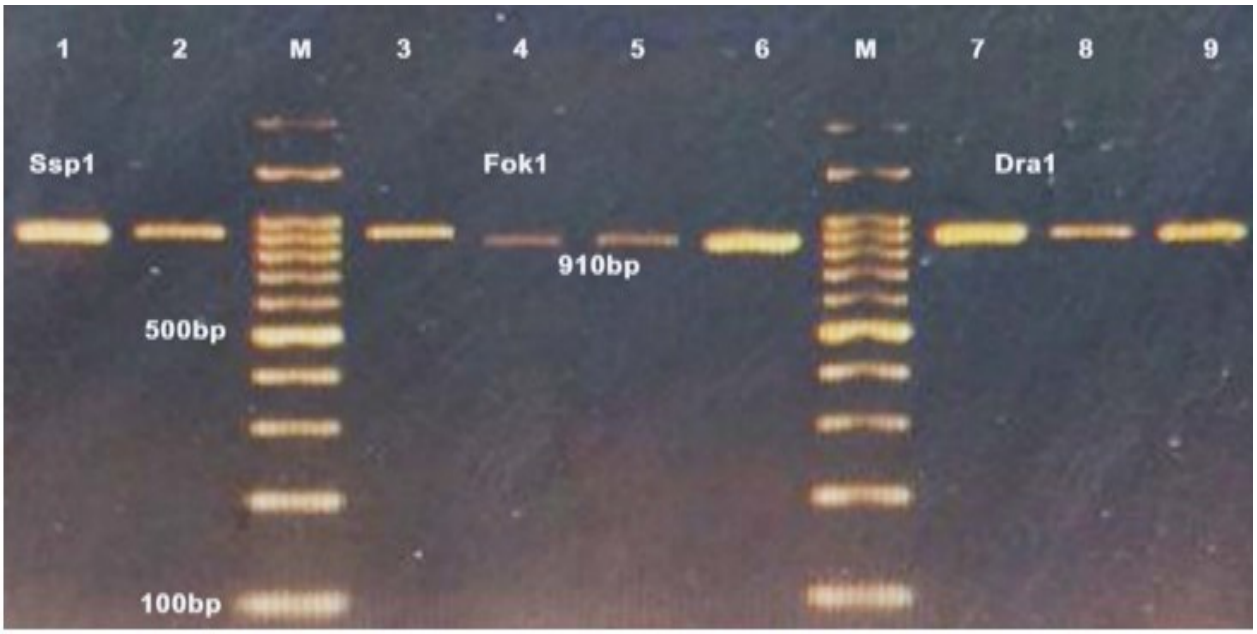

Fig. 2: PCR-RFLP analysis of Cox1 gene of *Sarcocystis*, demonstrating species-specific fragments of *S. cruzi*: Lane1and 2 digested whit Ssp1, Lane 4, 5 and 6 digested whit Fok1, Lane 8 and 9 digested whit Dra1, M marker of 100 bp, lane 3 and 7 uncut (control)

PCR-RFLP

For PCR-RFLP analysis, the *18S rRNA* and *Cox1* products of different *Sarcocystis* species were digested (19). Digestion of amplified PCR products was separately perfumed by three restriction enzymes: Dra1, Ssp1, and Fok1. Briefly, reaction mixtures were applied in a total volume of 50 µl, consisting of 10 units of restriction enzyme, 10-20 µl of PCR product, and 5 ml of buffer. All restriction mixtures were incubated for 16 h at 37 °C (Dra1, Ssp1) and 5 min at 37 °C (Fok1), followed by inactivation at 65 °C for 20 min (Dra1 and Ssp1) and at 65°C for 5 min (Fok1), (Thermo Scientific, USA). A 100-bp DNA ladder was utilized to estimate the sizes of the restriction fragments (Armanbiotech, cat no. YT8503).

DNA sequencing and analysis

The amplified PCR products of both genes were successfully sequenced for sampled *S. cruzi*. Assembly of DNA sequence on both strands was obtained from fragments consisting 773 and 858 consensus nucleotides of rRNA and *Cox1*, respectively. All Iranian *18S rRNA* and *Cox1* sequences were completely similar to each other as registered in GenBank under accession no. OP278729, and OP609867, respectively. The Iranian sequences were compared to eight corresponding sequences obtained from *18S rRNA* gene (AF176935, KT306827, KM434885, and KJ917944), and *Cox1* gene of *S. cruzi* (MT796928, MT796935, MW490605, KC209598). Based on the multiple alignments of Iranian *18S rRNA* (Fig. 3) and *Cox1* genes sequences with other *S. cruzi* strains (Fig. 4), different isolates were characterized by single nucleotide polymorphisms (SNPs) and indels in the present study.

According to accession numbers OP278729 and OP609867 (Figs 3, and 4), these sequencing results also showed nucleotide polymorphism in the sequenced *18S rRNA* and *Cox1* genes at positions 444, 459, 511, 518, 526, 527 and 539, as well as at 664 of the *18S rRNA* and 585, 599, 708, and 845 of the *Cox1*, re-

spectively. Sequencing of *18S rRNA* gene of the Iranian isolates demonstrated Sequencing of the Iranian isolates of *18S rRNA* demonstrated four nucleotide positions (444, 459, 539, and 664) are responsible for double peaks on the chromatogram. Two possible nucleotide characters were present for each of the four locations (Fig. 3); also, sequencing of *Cox1* revealed two nucleotide positions (585, and 845) responsible for double peaks, where two possible nucleotide characters were observed in each of these locations (Fig. 4).

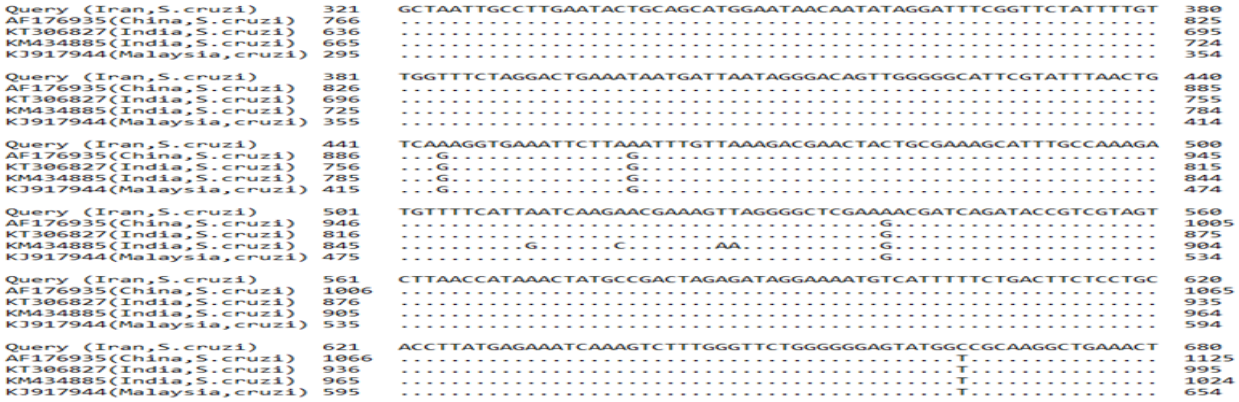

Fig. 3: A sequence alignment of the 18S rRNA (680 bp) derived from *S. cruzi* of the Iranian water buffalo and other *S. cruzi* strains. Nucleotide polymorphism in the sequenced 18S rRNA genes, at positions 444, 459, 511, 518, 526, 527, 539, and 664.

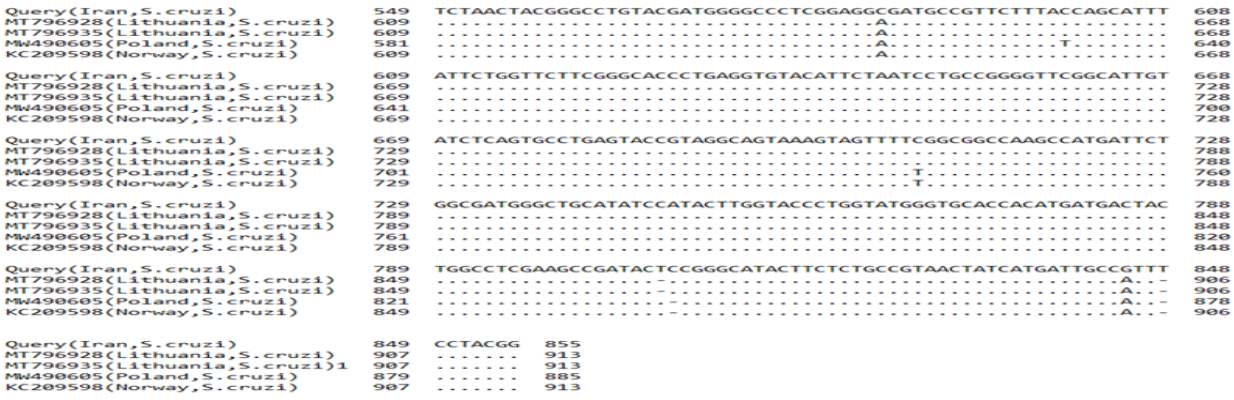

Fig. 4: A part of multiple sequence alignment of the Cox1 (855 bp) derived from *S. cruzi* of the Iranian water buffalo and other *S. cruzi* strains derived. Nucleotide polymorphism at positions 585, 599, 708, and 845

The phylogenetic analysis of *18S rRNA* and *Cox1* sequences by the maximum likelihood (ML) method (Fig. 5 and 6) exhibited that the Iranian isolates of *Sarcocystis 18S rRNA* sequences were in a group with three strains of *S. cruzi* (AF176935, KT306827 and KM434885), while *Cox1* sequences were grouped with two previously reported *S. cruzi* strains (MT796928 and MT796935). Furthermore, the cladogram of *18S rRNA* and *Cox1* genes demonstrated that these groups were more linked to *S. Levinei* (KU247921, and KU247920) and *S. cruzi* (MW490605), respectively.

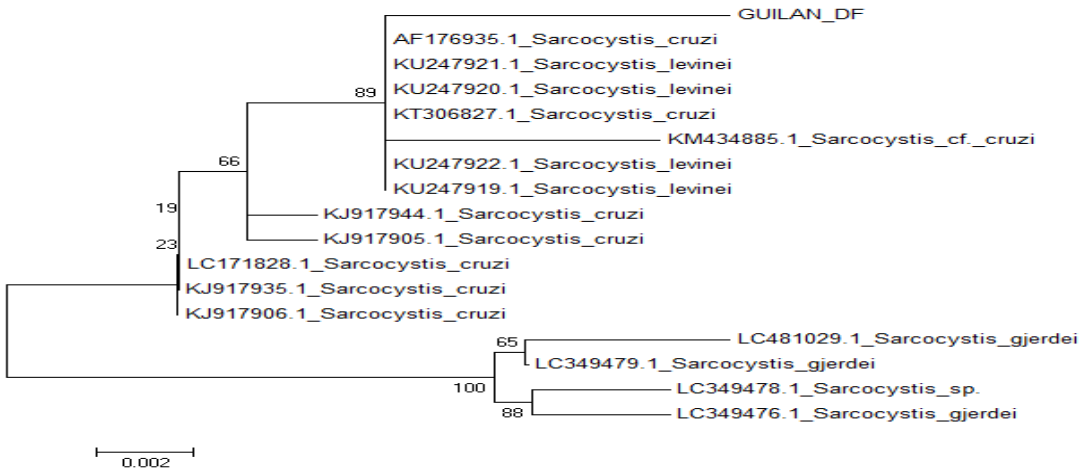

Fig. 5: Phylogenetic tree of partial 18S rRNA sequence of Iranian water buffalo strain and other *Sarcocystis* species based on maximum likelihood (ML) method. The scale bar indicates distance

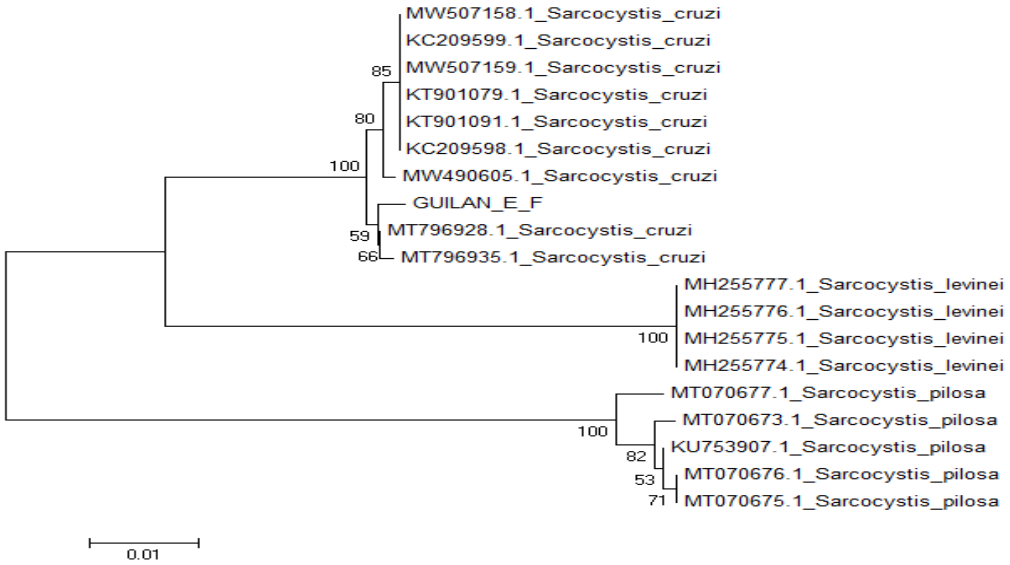

Fig. 6: Phylogenetic tree based on partial Cox1 sequence of Iranian water buffalo strain and other *Sarcocystis* species using maximum likelihood (ML) method. The scale bar indicates the distance

Discussion

Sarcocystis infection has been reported among vertebrates, including humans (23). The morphological evaluation of *Sarcocystis,* especially the structure of the cyst wall and sporocysts, has been used to identify *Sarcocystis* species in livestock. *Sarcocystis* may show quite a few variabilities, depending on the location and stage of development and other parameters of the parasite cell. Molecular investigations have been carried out for morphologically identified species (24). Today, available molecular methods are capable of identifying morphologically similar sarcocysts or belonging to other species (25). *Cox1* is the preferred target for taxonomic differentiation of *Sarcocystis* species affected ruminant intermediate hosts (e.g.,

cows, sheep, goats, deer, etc.), (26). Although *18S rRNA* sequences are mostly registered in public databases due to their frequent use for identification, the mitochondrial *Cox1* is the most useful tool for differentiating closely related *Sarcocystis* spp. in venomous mammals (27).

According to clinical, morphological, and pathological *characteristics, S. cruzi* has been identified in infected calves after experimentally infecting the calves with excreted oocysts of puppies (27). This protozoan may result in clinical manifestations such as weight loss, and abortion in calves. On the other hand, it probably causes nausea, stomachache, and diarrhea in humans after consuming half-cooked meat (28). Identification of *Sarcocystis* species has been performed through host characteristics, cyst morphology, and cyst wall ultrastructure, as well as molecular and biochemical features (29). To detect different species of *Sarcocystis* in meat, common techniques such as methylene blue staining, dob smear, and digestion have been applied in studies (30).

Serological methods have their own limitations in domestic animals such as significant antigenic similarity and subsequent cross-reactivity with other *Sarcocystis* species. In addition, serological tools are not useful for species identification (31). Molecular methods have been used to classify *Sarcocystis* spp. isolated from diverse samples (32). Highly conserved 18S ribosomal subunit variable regions are suitable for distinguishing *Sarcocystis* species in hosts. According to recent studies, *Cox1* is preferred for identifying *Sarcocystis* species in domestic animals (5, 33, 34). Based on the data presented herein, *S. cruzi* was found among buffaloes in Guilan Province, but no infection with other *Sarcocystis* species was found. *Sarcocystis* was reported mainly in the muscles of the esophagus, larynx, and tongue (35, 36). Our findings showed the highest rate of infection in the diaphragm. We utilized *18S rRNA* and *Cox1* genes to identify *Sarcocystis* sp. According to the gene sequence data collected by analysis of morphological criteria, it can be concluded that the tested isolates collected from water buffalo belong to *S. cruzi*.

The phylogenetic analyzes performed based on the *18S rRNA* gene showed a close evolutionary relationship between the current *S. cruzi* isolate and the China *S. cruzi* isolate (accession no. AF176935) (32), the Indian isolate (accession no. KT306827) (36) shown to have a little more than 99% sequence similarity with only five nucleotide substitution and 100% query coverage. Also, according to *Cox1* gene showed a close evolutionary relationship between the current *S. cruzi* isolate and the Lithuania *S. cruzi* isolate (accession no. MT796928, MT796935) (37), which was shown to have more than 99.48% sequence similarity with only two nucleotide substitution and 100% query coverage.

By comparing the recorded sequences, the polymorphisms of the *18S rRNA* and *Cox1*genes of the Iranian *S. cruzi* strain in our study may indicate geographical stability isolation. Intraspecific polymorphisms in these target genes can be usefully applied for phylogenetic assessment and the genetic structure of *S. cruzi*. Studying sarcocystosis from wider geographical areas and sequencing more loci, such as the rRNA large subunit gene, ITS1 or ITS2, can contribute to our deeper understanding of the differences between each species.

Sarcocystis isolates of sheep by 18S rDNA - RFLP and macroscopic cysts were reported to be *S. gigantea* and *S. arieticanis*, respectively (39). *S. cameli* was reported in camels for the first time in Iran based on an electron microscope and PCR-RFLP method (30). Available epidemiological data confirm the increase of sarcocystosis cases and the geographical spread of *Sarcocystis* in buffaloes of the Khuzestan province of Iran (2).

Buffalo breeding in Iran is mainly limited to the south and northwest regions. Sarcocystosis may be associated with decreased *production* of water buffaloes (10). Another study (2) revealed macroscopic forms of *Sarcocystis* in 3% of water buffaloes, while 83% exhibited microscopic forms. Our study is the first mo-

lecular description of *S. cruzi* in water buffalo in Iran. The findings of another study (19) are in accordance with the hypothesis that *S. cruzi* is capable of infecting water buffalo and is not limited to cattle. On the other hand, water buffalo were infected with *S. fusiformis, S. cruzi, S. hominis,* and *S. hirsute* based on RFLP of the *18S rRNA* gene. They reported that *Sarcocystis* spp. in infected cattle is capable of infecting water buffaloes (19).

Despite the presence of polymorphisms in the *18S rRNA* and *Cox1* variable regions of *S. cruzi*, complete identity for the FokI restriction site was determined in all strains from various regions, (i.e., China, India, Iran for *18S rRNA*; Iran and Lithuania for *Cox1*), suggesting the suitability of these conserved regions for FokI digestion as a suitable method for easy differentiation of *S. cruzi* from other species infecting water buffalo worldwide (Fig. 2 & 3).

Furthermore, all the double peaks in the chromatograms may be associated with at least two distinct variants of *18S rRNA* and *Cox1* genes in DNA isolated from cyst merozoites. The distinct variants might result from the *18S rRNA* and *Cox1* genes being a multicopy gene. They might be expressed in the *Sarcocystis* due to differences between gene copies from different merozoites. Unrelated double peaks for SNPs may be linked to the variants of the gene in various isolates of any global species.

However, changes in this gene can be studied directly by DNA sequencing, as has been done previously (6, 33), but this is *expensive* for identification and epidemiological research.

Jehle et al evaluated the partial sequence of the 18S rDNA by modified RFLP analysis to identify *Sarcocystis* spp. in cattle and water buffaloes in Vietnam. This method was called a cost-effective technique for identifying *Sarcocystis* species. In addition, the PCR amplicon sequence analysis of the collected samples was performed in comparison with the results of registered sequences. Different species such as *S. hirsute, S. cruzi,* and *S. hominis* were detected in cattle, while infection of water buffaloes with *S. fusiformis*, *S. cruzi, S. hominis,* and *S. hirsute* has been reported. Most *Sarcocystis* species of cattle were capable of infecting water buffalo (39).

Based on the molecular results of the present study, it seems that the microscopic forms are probably early stages of developing cysts of *S. cruzi* and are not different species of *Sarcocystis*.

Conclusion

This study is the first report of *S. cruzi* in water buffalo in Guilan Province North of Iran. Water buffalo could be considered as a replacement intermediate host for *S. cruzi*. In addition to the complete sequence of the *18S rRNA* gene and the mitochondrial *Cox1* gene, sequence analysis of other genetic loci will be useful to better identify the genotypes of different *Sarcocystis* species isolated worldwide.

Acknowledgements

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Conflict of interest

The authors declare that they have no conflicts of interest.

Data availability

The nucleotide sequence generated in the present study has been deposited in GenBank (https://www.ncbi.nlm.nih.gov/) under accession numbers OP278729 and OP609867.

References

1. Ayazian Mavi S, Teimouri A, Mohebali M, et al. *Sarcocystis* infection in beef and

industrial raw beef burgers from butcheries and retail stores: A molecular microscopic study. Heliyon. 2020; 6(6): e04171.
2. Oryan A, Ahmadi N, Mousavi SM. Prevalence, biology, and distribution pattern of *Sarcocystis* infection in water buffalo (*Bubalus bubalis*) in Iran. Trop Anim Health Prod. 2010; 42(10):1513–8.
3. Kirillova V, Prakas P, Calero-Bernal R, et al. Identification and genetic characterization of *Sarcocystis arctica* and *Sarcocystis lutrae* in red foxes (*Vulpes vulpes*) from Baltic States and Spain. Parasites Vectors. 2018; 11(1):173.
4. El-kady AM, Hussein NM, Hassan AA. First molecular characterization of *Sarcocystis* spp. in cattle in qena governorate, upper Egypt. J Parasit Dis. 2018; 42(1):114 –21.
5. Gjerde B, De la Fuente C, Alunda JM, et al. Molecular characterization of five *Sarcocystis* species in domestic sheep (Ovis aries) from Spain. Parasitol Res. 2020; 119(1):215–31.
6. Yang ZQ, Li QQ, Zuo YX, et al. Characterization of *Sarcocystis* species in domestic animals using PCR-RFLP analysis of variation in the *18S rRNA* gene: A cost-effective and simple technique for routine species identification. Exp Parasitol. 2002; 102(3-4):212–7.
7. Fayer R, Esposito DH, Dubey JP. Human infections with *Sarcocystis* species. Clin Microbiol Rev. 2015; 28(2): 295–311.
8. Dubey JP, Speer CA, Fayer R. Sarcocystosis in animals and man. 1st ed. CRC, Boca Raton, FL.1989a; 1–145.
9. Blagojevic B, Antic D. Assessment of the potential contribution of official meat inspection and abattoir process hygiene to biological safety assurance of final beef and pork carcasses. Food Control. 2014; 36:174–82.
10. Dubey JP, Speer CA, Shah HL. Ultrastructure of sarcocysts from water buffalo in India. Vet Parasitol. 1989b; 34(1-2):149–52.
11. Kalantari N, Bayani M, Ghaffari S. *Sarcocystis* cruzi: first molecular identification from cattle in Iran. Int J Mol Cell Med. 2013; 2(3): 125–30.
12. Borji H, Azizzadeh M, Kamelli M. A retrospective study of abattoir condemnation due to parasitic infections: economic importance in Ahwaz, southwestern Iran. J Parasitol. 2012; 98(5): 954–7.
13. Gjerde B, Josefsen TD. Molecular characterization of *Sarcocystis lutrae* n. sp. and *Toxoplasma gondii* from the musculature of two Eurasian otters (*Lutra lutra*) in Norway. Parasitol Res. 2015; 114(3):873–6.
14. Gjerde B. *Sarcocystis* species in red deer revisited: with a re-description of two known species as *Sarcocystis elongata n.* sp. and *Sarcocystis truncata n.* sp. based on mitochondrial *Cox1* sequences. Parasitol. 2014; 141(3):441–52.
15. Nourollahi Fard SR, Asghari M, Nouri F. Survey of *Sarcocystis* infection in slaughtered cattle in Kerman, Iran. Trop Anim Health Prod. 2009; 41(8):1633–6.
16. Verma SK, Lindsay DS, Grigg ME, et al. Isolation, culture and cryopreservation of *Sarcocystis* species. Curr Protoc Microbiol. 2017; 45: 20D.1.1-20D.1.2.
17. Chiesa F, Muratore E, Dalmasso A, et al. A new molecular approach to assess the occurrence of *Sarcocystis* spp. in cattle and products thereof: preliminary data. Ital J Food Saf. 2013; 2:148–51.
18. Al Qassab T, Shayan P, Kamkar Ab, et al. Reverse line blot hybridization assay as a suitable method for the determination of food adulteration in an example of sausage samples. European Food Research and Technology. 2019; 245:1677-83.
19. Li QQ, Yang ZQ, Zuo YX, et al. A PCR-based RFLP analysis of *Sarcocystis cruzi* (Protozoa: Sarcocystidae) in Yunnan province, PR China, reveals the water buffalo (*Bubalus bubalis*) as a natural intermediate host. J Parasitol. 2002; 88(6):1259–61.
20. Larkin MA, Blackshields G, Brown NP, et al. ClustalW and Clustal X version 2.0. Bioinformatics. 2007; 23(21):2947–8.
21. Tamura, K, Peterson D, Peterson N, et al. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol. 2011; 28(10):2731–9.
22. Abdel-Ghaffar F, Mehlhorn H, Bashtar AR, et al. Life cycle of *Sarcocystis camelicanis*

infecting the camel (*Camelus dromedarius*) and the dog (*Canis familiaris*), light and electron microscopic study. Parasitol Res. 2009; 106(1):189–95.
23. Kia E B, Mirhendi H, Rezaeian M, et al. First molecular identification of *Sarcocystis meischeriana* (Protozoa, Apicomplexa) from wild boar (*Sus scrofa*) in Iran. Exp Parasitol. 2011; 127(3): 724–6.
24. Dahlgren S, Gjerde B. Genetic characterization of six *Sarcocystis* species from reindeer (Rangifer tarandus tarandus) in Norway based on the small subunit rRNA gene. Vet Parasitol. 2007; 146(3-4):204–13.
25. Gjerde B. Phylogenetic relationships among *Sarcocystis* species in cervids, cattle and sheep inferred from the mitochondrial cytochrome c oxidase subunit I gene. Int J Parasitol. 2013; 43(7): 579–91.
26. Gjerde B. Molecular characterisation of *Sarcocystis bovifelis, Sarcocystis bovini n.* sp., *Sarcocystis hirsuta* and *Sarcocystis cruzi* from cattle (Bos taurus) and *Sarcocystis sinensis* from water buffaloes (*Bubalus bubalis*). Parasitol Res. 2016; 115(4):1473–92.
27. Naghibi A, Razmi G, Ghasemifard M. Identification of *Sarcocystis cruzi* in cattle by using of experimental infection in final and intermediate hosts (In Persian). J Fac Vet Med Univ Tehran. 2002; 57:67–9.
28. Pescador CA, Corbellini LG, Oliveira ECD, et al. Aborto ovino associado com infecção por *Sarcocystis* sp. Pesquisa Veterin´aria Brasileira. 2007; 27(10):393–7.
29. Motamedi GR, Dalimi A, Nouri A, et al. Ultrastructural and molecular characterization of *Sarcocystis* isolated from camel (*Camelus dromedarius*) in Iran. Parasitol Res. 2011; 108(4): 949–54.
30. Holmdahl OJ, Mattsson JG, Uggla A, et al. Oligonucleotide probes complementary to variable regions of *18S rRNA* from *Sarcocystis* species. Mol Cell Probes. 1993; 7(6):481–6.
31. Mor´e G, Bacigalupe D, Basso W, et al. Serologic profiles for *Sarcocystis* sp. and *Neospora caninum* and productive performance in naturally infected beef calves. Parasitol Res. 2010; 106(3):689–93.
32. Yang Z, Zuo YX, Yao YG, et al. Analysis of the *18S rRNA* genes of *Sarcocystis* species suggests that the morphologically similar organisms from cattle and water buffalo should be considered the same species. Mol Biochem Parasitol. 2001; 115(2):283–8.
33. Ellis JT, Luton K, Baverstock PR, et al. Phylogenetic relationships between *Toxoplasma* and *Sarcocystis* deduced from a comparison of 18S rDNA sequences. Parasitology. 1995; 110(5): 521–8.
34. Oryan A, Moghaddar N, Gaur SNS. The distribution pattern of *Sarcocystis* species, their transmission, and pathogenesis in sheep in the Fars Province of Iran. Vet Res Commun. 1996; 20(3): 243–3.
35. Heckeroth AR, Tenter AM. Comparison of immunological and molecular methods for the diagnosis of infections with pathogenic *Sarcocystis* species in sheep. The Tokai J Exp Clin Med. 1998; 23(6):293–302.
36. Daptardarkar M, Singh BB, Gill JPS, et al. Prevalence and first molecular identification of *Sarcocystis* species in cattle and water buffaloes in Punjab, India. Acta Parasitol. 2016; 61(3): 523-8.
37. Prakas P, Strazdaite-Zieliene Z, Januskevicius V, et al. Molecular identification of four macrocystis species in cattle from Lithuania, including *S. hominis*, and development of a rapid molecular detection method. Parasit Vectors. 2020;13(1):610.
38. Dalimi A, Paykari A, Esmaeilzadeh M. Identification of *Sarcocystis* species of infected sheep in Ziaran abattoir, Qazvin, using PCR-RFLP (In Persian). Modares Med Sci. 2008; 11:65–72.
39. Jehle C, Dinkel A, Sander A. Diagnosis of *Sarcocystis* spp. in cattle (*Bos Taurus*) and water buffalo (*Bubalus bubalis*) in northern Vietnam. Vet Parasitol. 2009; 166(3-4):314–32.