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Original article

MOLECULAR IDENTIFICATION OF *SARCOCYSTIS CRUZI* ISOLATED FROM IRANIAN BUFFALOES IN GUILAN PROVINCE

F. DAMESHGHI[1], S. SHIRALI[1,2], P. SHAYAN[3] & B. SHEMSHADI[1]

[1]Department of Pathobiology, Science and Research Branch, Islamic Azad University, Tehran, Iran; [2]Department of Biotechnology, Ahvaz Branch, Islamic Azad University, Ahvaz, Iran; [3]Department of Pathobiology, Faculty of Veterinary Medicine, University of Tehran, Iran

Summary

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Sarcocystis species are prevalent in Iran and have economic and public health consequences on animals and humans. The present study aimed at molecular identification of *Sarcocystis* spp. isolated from Iranian buffaloes in Guilan province based on 18s ribosomal RNA (*18s rRNA*) and cytochrome oxidase subunit I (*Cox1*) sequences. For this purpose, a total of 100 buffalo carcasses were sampled from four tissues (oesophagus, diaphragm, shoulder, thigh), and assessed microscopically for sarcocysts. Samples were suspected of being infected with *Sarcocystis* spp. according to morphological evaluation. Amplification of *18s rRNA* and *Cox1* sequences were performed from DNA extracted from any detected *Sarcocystis*. The phylogenetic tree was plotted by Neighbor-Jinning (NJ) phylograms. Phylogenetic analysis of *18s rRNA* and *Cox1* genes showed no differences between *Sarcocystis* isolates of the same species and a low level of genetic variability was found among isolates and other *Sarcocystis cruzi* sequences. *S. cruzi* was identified as the species of Iranian buffalo in the Guilan province of Iran. This is the first study of the *Cox1* gene and *18s rRNA* sequences to confirm the presence of *S. cruzi* in Iranian buffaloes.

Key words: *18s rRNA, Cox1*, Iranian buffaloes, *Sarcocystis* spp.

INTRODUCTION

Sarcocystis spp. are two-hosts coccidia that have a wide range of hosts (Fayer, 2004). Life cycle is characterised by asexual multiplication in intermediate hosts (e.g., herbivores and omnivores) (Lindsay *et al*., 2017) and sexual multiplication in definitive hosts including cervids (dogs, wolves, and cats) (Chen *et al*., 2011). Symptoms induced by some *Sarcocystis* species are associated with haemorrhagic diathesis and encephalitis in intermediate hosts (Kolenda *et al.,* 2014), whereas

acute pregnancy infection with *Sarcocystis* species can lead to foetal death, and abortion (Dubi *et al*., 1989).

Macroscopic observations have reported that the most infected buffalo organs with the *Sarcocystis* parasite were the oesophagus, diaphragm, tongue, and heart (Dong *et al.,* 2018; Morsy *et al*., 2018).

Sarcocystosis is related to economic losses in livestock industries (Xiang *et al*., 2009), where chronic infections have been attributed to the reduced quality of livestock meat (Fayer & Elsasser, 1991).

Buffaloes are intermediate hosts of several *Sarcocystis* species including *S. fusiformis*, *S. cruzi*, *S. levinei*, *S. sinensis*, and *S. hominis* (Li *et al*., 2002). Among these species, the cyst of *S. fusiformis* is morphologically distinguished from the others by its long spindle shape (length: 5–35 mm) (Gjerde *et al*., 2015). *S. fusiformis* has been identified in water buffaloes of Egypt by *18s rRNA*, *28s rR*NA, *Cox1*, and *ITS1* gene sequences, but only by *18s rRNA* in countries such as Vietnam, Iran, and China (Oryan *et al*., 2011; Hu *et al*., 2016). Identification and description of phylogenetic relationships, which are inferred from phenotypic traits, are often not accurate enough. A combination of morphological and biological findings with molecular data resulting from methods such as isozyme analysis can be useful (O'Donoghue *et al*., 1986).

18s rRNA, *28s rRNA*, *ITS*, and *Cox1* sequences have been reported to be capable of distinguishing *Sarcocystis* (Gjerde & Josefsen, 2015). In China, the *18s rRNA* gene sequencing was used to detect *S. fusiformis* in buffaloes (Li *et al*., 2002). However, the *Cox1* sequence is described to be a favourable marker compared with *18S rRNA* as *Cox1* genes are capable of distinguishing *Sarcocystis* species (Gjerde, 2014).

The present study aimed at identifying *Sarcocystis* species in Guilan province, Iran based on morphological and molecular evaluation.

MATERIALS AND METHODS

Sample source

The samples were obtained from infected water buffaloes in the Rasht Slaughterhouse, Guilan Province, North of Iran from January to May 2022. The oesophagus, diaphragm, shoulder, and thigh muscles of each buffalo were sampled.

The washed cysts were examined for morphological characteristics and then kept at −20 °C until DNA extraction (Cabaj *et al*., 2020).

DNA extraction and amplification of 18S rRNA and Cox1 genes

DNA extraction was done with a genomic DNA kit (MBST.IRAN) according to the manufacturer's instructions. Targeted genes were amplified using *18s rRNA* gene (F: 5-TCAGGGAGGTAGTGACA AGA-3; R: 5-ATGTCTGG ACCTGGT GAGTT-3) and *Cox1* gene primers (F: 5-CTTTAGCGTTGTTGGTAC-3; R: 5-CCCGTAGGAATGGCAAT-3).

18S rRNA (JQ713824) and *Cox1* primers (KU247899) were synthesised based on the registered sequences at Sinaclon Biotech Co, Tehran, Iran. PCR was performed by the standard method, except for setting the denaturation temperature at 94 °C. The PCR amplicons were assessed by electrophoresis in 1.5% agarose gel. The nucleotide sequences were finally sent to Takapozist Biotech Company in Iran for sequencing.

Sequencing and phylogenetic analyses

A total of four PCR products (two products from each gene) were sent for sequencing (Takapo Zist, Iran). The sequences were then matched against the BLAST database sequences. Clustal W V.2 software was applied to align gene sequences (Larkin *et al.,* 2007). MEGA 6.06 software was utilised to generate Neighbor-Jinning (NJ) phylograms (with 1000 replicates) of gene sequences. (Tamura *et al*., 2011).

RESULTS

Tissue samples from buffaloes were macroscopically assessed for focal or diffuse lesions with a grey discoloration. Small spindle shape sarcocysts (1.0×0.1 mm) with hair-like protrusions were observed, indicating the distinct characteristics of *Sarcocystis cruzi. S. cruzi* was small in size, not more than 6 mm in length.

Our phylogenic tree exhibited that the sarcocysts were identical to the *S. cruzi*, while other species including *S. levinei, S. sinensis, S. fusiformis,* and *S. hominis* were not discovered in the present study. *S. levinei, S. fusiformis,* and *S. sinensis* are considered as most common species reported in buffaloes, whereas *S. cruzi* and *S. hominis* are mostly identified in cattle, and rarely detected in buffaloes. *S. cruzi* is considered to be the most common species reported in cattle in Belgium and

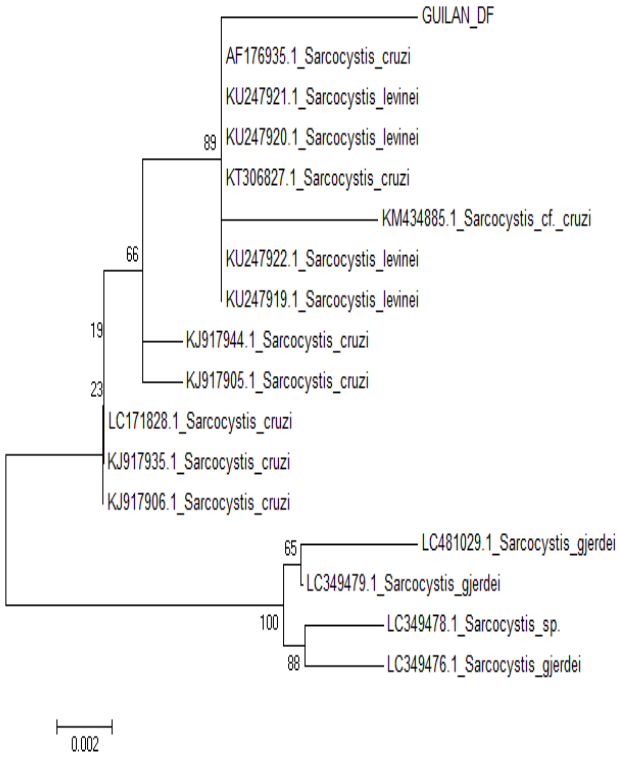

Fig. 1. Phylogenetic analysis of partial *18s rRNA* sequences of *Sarcocystis* using the NJ method. The new sequence of *S. cruzi* is named GUILAN_DF (accession number OP278729).

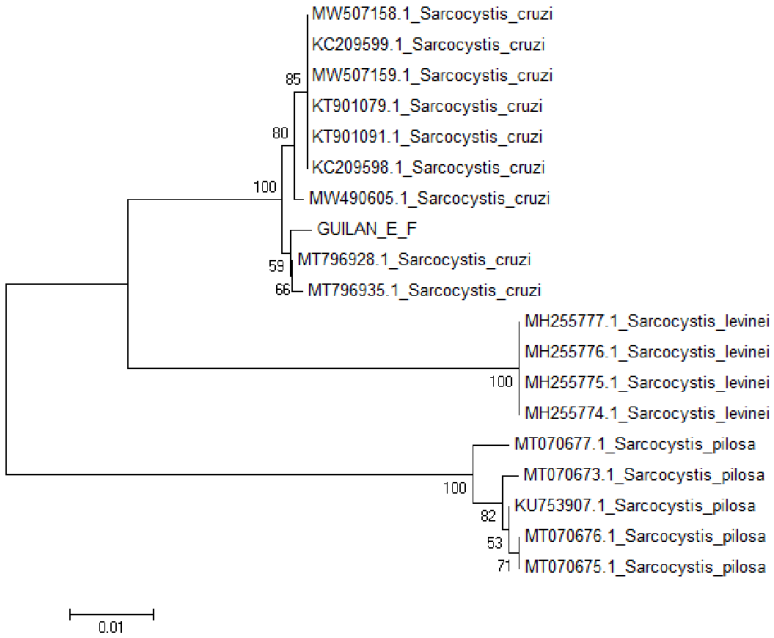

Fig. 2. Phylogenetic analysis of partial *Cox1* sequences of *Sarcocystis* using the NJ method. The new sequence of *S. cruzi* is named GUILAN_E_F (accession number OP609867).

Netherlands in the last decade. In this research, *Sarcocystis cruzi* was detected using two genes *18s rRNA* and *Cox1* in buffalo carcasses in northern Iran.

Genomic DNA samples of sarcocysts were used as templates for the amplification of the *18s rRNA* gene with a length of 860 bp. Our sequence was aligned with registered sequences of *18s rRNA* gene, such as *S. cruzi* (AF176935, KT306827, KM434885, and KJ917944), *S. levinei* (KU247921, KU247920), and *S. gjerdei* (LC481029) with an aid of Clastal W v.2 software. The phylogenetic analysis was visualised by plotting the phylogenic trees generated by the NJ method and adjusting the bootstrap method as a test. The phylogeny tree of *18s rRNA* gene sequences is visualized (Fig. 1), where the amplified sequence (GUILAN_DF) showed the same branch as the *S. cruzi 18s rRNA* gene sequences. Our study applied genomic DNA samples as a template to amplify *Cox1* sequences of 950 bp length. By Clustal W v.2 software, amplified *Cox1* sequence was aligned with registered sequences such as *S. cruzi* (MT796928, MT796935, MW490605, and KC209598), *S. levinei* (MH255776, MH255774, and MH255777), and *S. pilos*a (MT070677, and MT070673). The phylogeny of *Cox1* gene sequences (named GUILAN_E_F) exhibited the same branch of other sequences of *S. cruzi* (Fig. 2).

DISCUSSION

There are different types of molecular research to diagnose *Sarcocystis* in buffalo worldwide (Latif *et al*., 2013; Gjerde *et al*., 2015), among which *18s rRNA* and *28s rRNA* are extremely conserved and

are commonly used to discern closely related species, while the relatively fast evolution of *ITS* has caused *ITS* to be used less than others (Kolenda *et al*., 2014). In addition, the *Cox1* is phylogenetically able to better distinguish different species when compared with the *18s rRNA* gene sequence (Dubey *et al*., 1989). In the past decades, *18s rRNA* has been sued to demonstrate the presence of *Sarcocystis* in animals, including buffaloes (Gjerde & Johan, 2014). *28s rRNA* and *18s rRNA* gene sequences were applied for detecting *Sarcocystis* in goats in China, while only *18s rRNA* has been used for the detection of *Sarcocystis* in buffaloes (Li *et al*., 2002; Hu *et al*., 2016). In the African continent, *18S rRNA*, *28S rRNA*, *Cox1*, and *ITS* gene sequences were applied for identifying the par*asite* in the *Sarcocystis* outbreak (Gjerde *et al*., 2015). Furthermore, *18s rDNA* amplicons have been previously applied for the detection of parasites such as *Giardia intestinalis, T. gondii*, *Entamoeba histolytica*, etc. (Moreno *et al.,* 2018). *18s rRNA* gene is another marker for the detection of specific *Sarcocystis* species (Murata *et al*., 2018). In the current study, both *18s rRNA* and *Cox1* sequence was applied as a supplement to investigate *S. cruzi* in the buffaloes of Gilan Province, Iran.

Phylogenetic trees of *18s rRNA* and *Cox1* genes showed a higher level of identity between the sample and *S. cruzi* sequences published in NCBI. Four registered sequences of *S. cruzi* were used (Fig. 1), where the sequences from Yunnan, China (AF176935) and Ludhiana, India (KT306827) were most closely related to the GUILAN_DF sequence, respectively, while the India Punjab sequence (KM434885) showed less similarity. This result showed that *18s rRNA* analysis was capable of distinguishing *S. cruzi* sequences from Yunnan, China and Guilan, Iran, but was unable to genetically distinguish *S. cruzi* sequences from Yunnan, Guilan and Punjab, India. Another effective factor is the insufficiency of collected samples, which can be completed in subsequent studies. Three registered sequences of *S. cruzi* from Lithuania (MT796928 and MT796935), Poland (MW490605), and our sequence (GUILAN_E_F) were found to be in two clusters. This finding revealed that the *Cox1* gene sequence was able to differentiate the *S. cruzi* sequence from Lithuania, Poland, and Guilan (Iran).

Our results demonstrated that the identified sequences GUILAN_DF and GUILAN_E_F (OP278729 and OP609867) derived from Iranian buffaloes are similar to the *Cox1* and *18s rRNA* gene sequences of *S. cruzi* isolated from cattle, indicating that cattle and water buffalo can be intermediate hosts of these species. Therefore, they are not limited to any host.

CONCLUSION

Overall, *S. cruzi* was confirmed as the commonest protozoan species of Iranian buffaloes in the Guilan province of Iran. *18s rRNA* and *Cox1* genes can effectively distinguish *Sarcocystis* species from Iranian buffaloes. This is the first report of the *Cox1* gene to confirm the infection of *S. cruzi* in Iranian buffaloes. The findings of the present research can help improve the molecular data of *Sarcocystis* species and their identification in Iran.

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Correspondence:

Saloomeh Shirali
Department of Biotechnology,
Ahvaz Branch, Islamic Azad University,
Ahvaz, Iran
yel: +98 9166170960
e-mail: s.shirali2017@gmail.com