

MOLECULAR IDENTIFICATION OF SARCOCYSTIS SPECIES IN BOVINE MINCED MEAT USING PARTIAL CYTOCHROME OXIDASE SUBUNIT 1 (COX1) GENE IN VAN PROVINCE, TURKEY

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ABSTRACT

Sarcocystis species are obligate two-host protozoan parasites classified in the phylum Apicomplexa. Cattle are generally accepted to be the intermediate hosts for seven species, i.e., *S. cruzi*, *S. hirsuta*, *S. hominis*, *S. bovifelis*, *S. bovine*, *S. rommeli*, and *S. heydorni*. Since it is not possible to differentiate between some species using amplification of the 18S ribosomal (rRNA) gene, the aim of this study was to reveal the molecular characterization of *Sarcocystis* species obtained from cattle minced meat amplifying partial cytochrome oxidase subunit 1 (Cox1) gene. Fifty DNA samples were used. Sequence analyses of amplicons belonging to positive isolates were performed and their phylogenetic structures were investigated. While *S. hominis* was found in only one sample, it was molecularly confirmed that *S. bovifelis* was dominant species in other samples. We designed primer set in present study could not differentiate between *S. bovine*, *S. rommeli* and *S. bovifelis* species. Phylogenetic analyses of isolates with GenBank records (OK041347-OK041353) were performed with similar isolates in the world. According to phylogenetic analysis, sequence of *S. bovifelis* (OK041347) was found closer to the isolates from cattle skeletal muscle in Argentina (KT900970 and KT900962). *S. hominis* (OK041352) isolate showed high genetic similarity to isolates from Netherlands and Italy (MK497840; MH021119). In conclusion, genetic characterization of *S. bovifelis* and *S. hominis* was performed for the first time in Van province of Turkey by partial cytochrome oxidase subunit 1 (Cox1) gene.

Key words: : sarcocystis, cox1, cattle, PCR, Van, Turkey.

Introduction

Sarcocystis species are protozoan parasites, with an obligatory two-host cycle and classified in the phylum Apicomplexa. Over 220 species were identified in the genus *Sarcocystis* and widely seen across the world (Prakas and Butkauskas, 2012; Oğuz et al. 2021). *Sarcocystis* spp. heteroxen and protozoan parasites form cysts in tissues of intermediate hosts and are thrown out as sporocysts with definitive hosts. *Sarcocystis* species are obligate two-host protozoan parasites classified in the phylum Apicomplexa. Although there has recently been confusion regarding the validity and classification of several *Sarcocystis* spp., cattle are generally accepted to be the intermediate hosts for seven species, i.e., *S. cruzi*, *S. hirsuta*, *S. hominis*, *S. bovifelis*, *S. bovine*, *S. rommeli*, and *S. heydorni*. (Hu et al. 2017; Prakas et al. 2020; Rubiola et al. 2021). Canids (*S. cruzi*), felids (*S. hirsuta* and *S. rommeli*) and humans (*S. hominis* and *S. heydorni*) are reported as the definitive hosts of these species. In addition, it is reported that the definitive host of *S. bovine* and *S. bovifelis* species is cats (Dubey, 2015; Gjerde, 2016; Hu et al. 2017; Rubiola et al. 2018). It is very important to be able to make precise species identifications of this genus, which also includes zoonotic species. There are morphologically indiscriminable species in the *Sarcocystis* genus. For this reason, species identification has been applied by molecular technique in recent years (Gjerde, 2016; Hoeve-Bakker et al. 2019; Rubiola et al. 2019; Murata et al. 2018; Prakas et al. 2020; Oğuz et al. 2021; Rubiola et al. 2021).

It has been reported that the 18S rRNA fragment and *S. hominis*, *S. bovifelis*, *S. bovini* and *S. rommeli* can not be discrimination and the term ‘‘*S. hominis*-like’’ should be used for all of them (Rubiola et al. 2018). There is considerable confusion about the validity and classification various *Sarcocystis* spp. in cattle. Researchers can identify *S. bovifelis*, *S. hominis*, *S. cruzi*, *S. rommeli* and *S. hirsuta* using Cox1 gene (Rubiola et al. 2019; Rubiola et al. 2020; Prakas et al. 2020; Rubiola et al. 2021). Moreover, it has been reported that this gene region is more preferable in differentiate taxonomically related *Sarcocystis* species (Gjerde, 2013; Prakas et al. 2020). It is necessary to establish a accurate diagnosis of species in order to evaluate the public health problems arising from the consumption of contaminated beef. In the previous work (Oğuz et al. 2021), there are samples could not identify *Sarcocystis* spp. in cattle minced meat obtained from various butcher shops and markets in Van, Turkey. The aim of the present study was to molecular identification of *Sarcocystis* species obtained from cattle minced meat using partial cytochrome oxidase subunit 1 (Cox1) gene.

Materials and methods

During 2019, a number of 150 meat sample of cattle were collected from various butcher shops and markets in Van. Fifty samples whose species could not be identified using the 18S rRNA gene were selected (Oğuz et al. 2021). Then, they were stored at -20°C for partial cytochrome oxidase subunit 1 (Cox1) gene analysis. Genomic DNA was initially subjected to PCR with the forward primer F1: 5'- TGTACATACTTACGGCAGGT-3' and reverse R1: 5'- CCGTAGGTATGGCGATCAT-3' specific primers that amplify approximately 850 bp for the Cox1 gene (Rubiola et al. 2018). PCR was performed in a final volume of 25 µl containing 10 µL of 5X MyTaq Reaction buffer (taq polymerase free), 10 pmol 1 µL of each primer, 0.5 µL Taq DNA polymerase (1.25 IU) (MBI Fermentas, Lithuania), 5 µL genomic DNA and 7.5 µL DNase- and RNase-free sterile distilled water (Biobasic, Canada). The cycling conditions began with one cycle at 94° C for 5 min, followed by 35 cycles at 94° C for 1 min, 60°C for 45 s, 72°C for 30 s and ending with one cycle at 72° C for 5 min. PCR products (10 µl) were subjected to electrophoresis on 1.5% agarose gel and visualized with the gel documentation system. PCR products were sent to Sentebiolab biotechnology company in Ankara for sequencing. The final consensus sequences of the isolates were analyzed in the GenBank database "nucleotide BLASTn" (National Center for Biotechnology Information, www.ncbi.nlm.nih.gov/BLAST) and similarity rates were compared with the isolates reported from different countries.

For the samples whose accurate identification or sequencing results could not be obtained at the initial PCR step were subjected to the PCR by using the different specific primers; COI_HB: AATGTGGTGCGGTATGAACT and COI_H: GGCACCAACGAACATGGTA (amplifying ~420 bp) for *S. hominis*; COI_HB: AATGTGGTGCGGTATGAACT and COI_B: TCAAAAACCTGCTTTGCTG (amplifying ~700 bp) for *S. bovifelis* (Rubiola et al. 2020). *Sarcocystis* spp. (*bovini/rommeli/bovifelis?*), which amplifies approximately 230 bp from the Cox1 gene, BOVF: GGTTTTGCCAGAATCAACG and BOVR: CGCCGTACCCAGGAAGTTGA were subjected to PCR with the original specific primers. PCR was performed in a final volume of 25 µl containing 10 µL of 5X MyTaq Reaction buffer (taq polymerase free), 10 pmol 1 µL of each primer, 0.5 µL Taq DNA polymerase (1.25 IU) (MBI Fermentas, Lithuania), 5 µL genomic DNA and 7.5 µL DNase- and RNase-free sterile distilled water (Biobasic, Canada). The cycling conditions began with one cycle at 95° C for 3 min, followed by 35 cycles at 95° C for 1 min, 58° C for 1 min and 72°C for 30 s and ending with one cycle at 72° C for 5 min. The products (10 µl) obtained at the end

of PCR were subjected to electrophoresis at 100 volts for 1 hour on 1.5% agarose gel and visualized with the gel documentation system. The identification was made by considering the band sizes in the reference manuscript (Rubiola et al. 2020). Primers for *Sarcocystis* spp. (*bovini/rommeli/bovifelis*?) were designed and determined on the partial cytochrome oxidase subunit 1 (Cox1) gene region of the isolate registered as Genbank accession number: LC171858. It was then synthesized in a commercial company (Sentebiolab, Ankara).

Sanger sequencing

The sequence chromatograms were controlled and arranged using the BioEdit software (Hall 1999). The final consensus sequences of our isolates were subjected to the “BLAST analysis” (<http://www.ncbi.nlm.nih.gov/BLAST>) and their similarity rates were compared with the isolates reported from different countries. Genetic distances were calculated using the Kimura 2 parameter model in MEGA 7.0 (Kumar et al. 2016). The phylogenetic analysis and the construction of phylogenetic tree were performed with 1000-repeated bootstrap using “maximum likelihood (ML)” and 2500-repeated bootstrap using “Neighbor-Joining” methods in the MEGA 7.0 (Kumar et al. 2016) software. The nucleotide sequences obtained in the study were submitted as the corresponding accession numbers of OK041347-OK041353 in GenBank.

Results

Expected fragments of ~850 bp for *Sarcocystis* spp., ~420 bp for *S. hominis*, ~700 bp for *S. bovifelis* and ~230 bp for *Sarcocystis* spp. (*bovini/rommeli/bovifelis*?) were successfully amplified. *Sarcocystis* spp. (~850 bp) was determined in all positive DNA samples (Figure 1). Moreover, *S. bovifelis* was found in 41 samples (Figure 2), whereas *S. hominis* was detected in only one samples (Figure 3). As a result of the *Sarcocystis* spp. (*bovini/rommeli/bovifelis*?) primer, 32 samples were found positive bands (Figure 4). According to sequence results, 37 readable results were obtained, while 13 of our samples did not result in readable sequences. The sequence results of 32 isolates from which 850 bp and 700 bp bands were obtained were subjected to comparison analysis in the GenBank database, and it was determined that they were 99% similar to *Sarcocystis bovifelis* (accession numbers: KT900962 and KT900970) (Figures 5 and 6). Our *S. hominis* (OK041352) isolates was found to be 99% similar to (accession number: MH021119) from Italy (Figure 7). The sequence results of four isolates with bands around 230 bp showed 100% homology with *S. bovini* (accession number: KT901022) and *S. rommeli* (accession number: KY120292) (Figure 8). These isolates also displayed a high identity of 97% with *S. bovifelis* (accession number: KT900976). In this sense, the identifying the types as *S. bovini*, *S. rommeli* and *S. bovifelis* could not be determined with original primer set designed in this study. The reason for the accurate unidentified species can be attributed to the shortness of the amplified region, the low specificity of the primer set we designed, the low genetic difference between three species and the possibility of mixed infections.

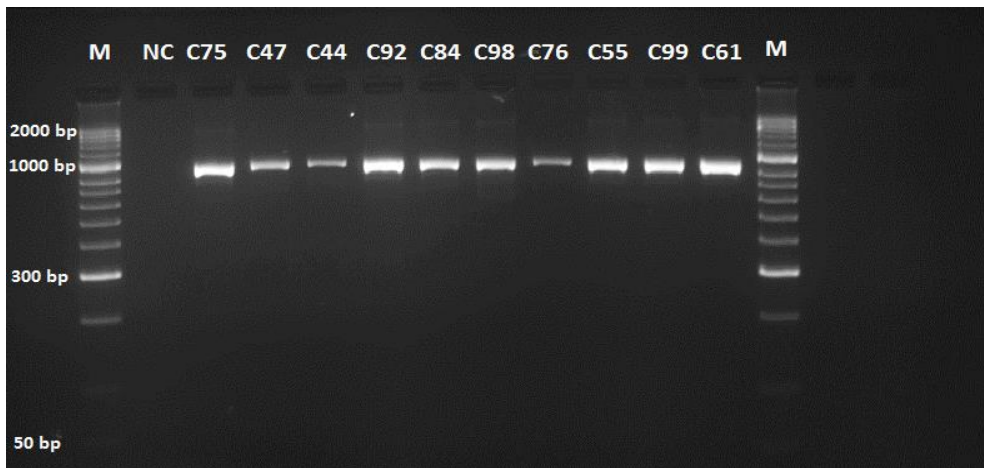


Figure 1: The single-PCR products of *Sarcocystis* species. M: 50-2000 bp molecular size marker, NC: Negative PCR control; C75, C47, C44, C92, C84, C98, C76, C55, C99, C61: *Sarcocystis* spp. positives (~850 bp).

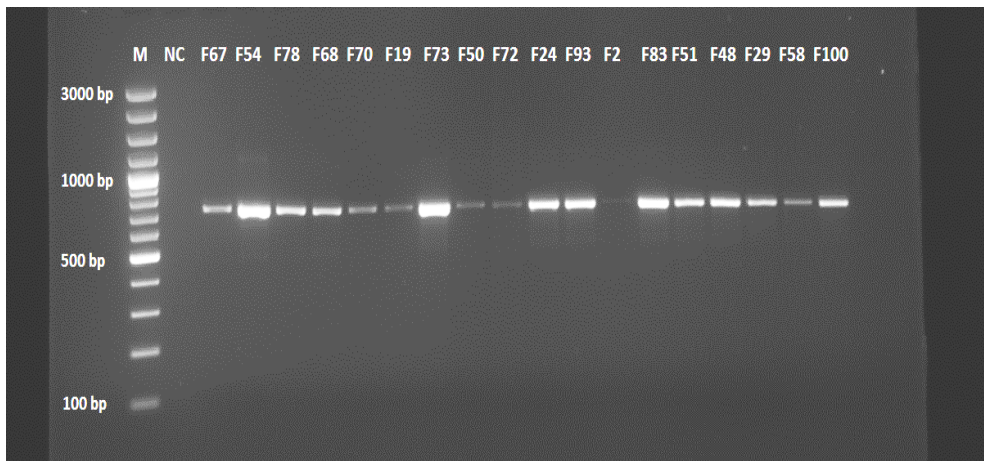


Figure 2: The single-PCR products of *Sarcocystis bovifelis*. NC; Negative PCR control

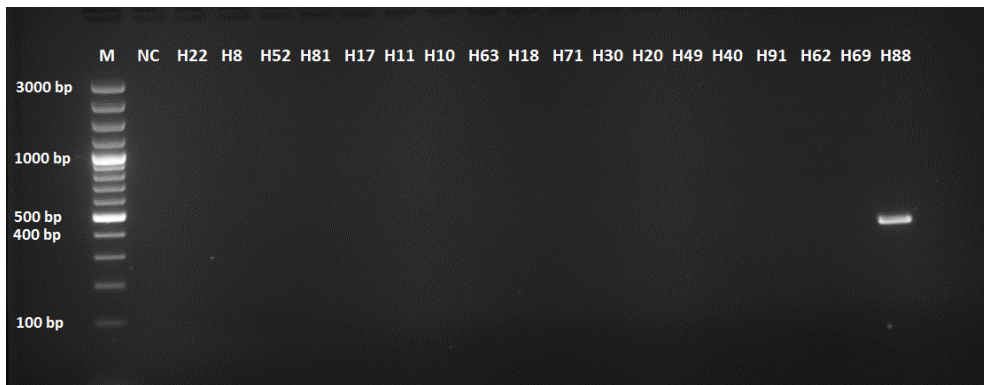


Figure 3: The single-PCR products of *Sarcocystis hominis*. M; 100-3000 bp marker, NC; Negative PCR control, Positive sample; H88. Negative samples; H22, H8, H52, H81, H17, H11, H10, H63, H18, H71, H30, H20, H49, H40,

H91, H62, H69.

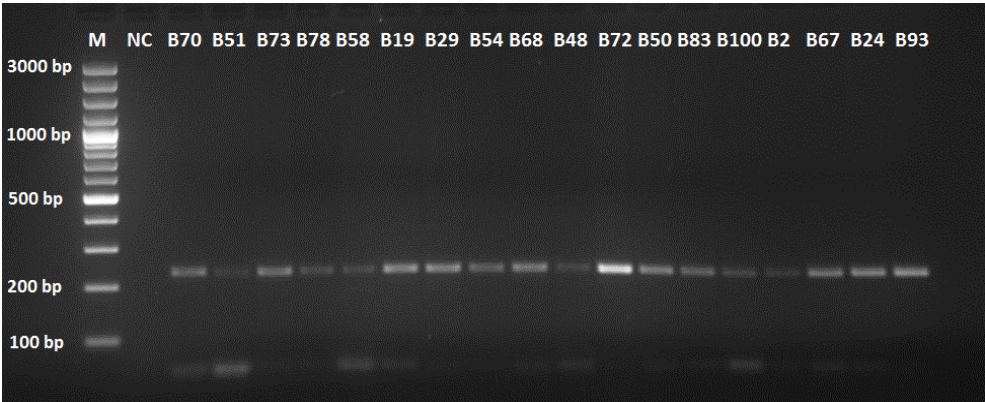


Figure 4: The single-PCR products of *Sarcocystis* species. M: 100-3000 bp molecular size marker, NC: Negative PCR control; B70, B51, B73, B78, B58, B19, B29, B54, B68, B48, B72, B50, B83, B100, B2, B67, B24, B93: *Sarcocystis* spp. positives (~230 bp).

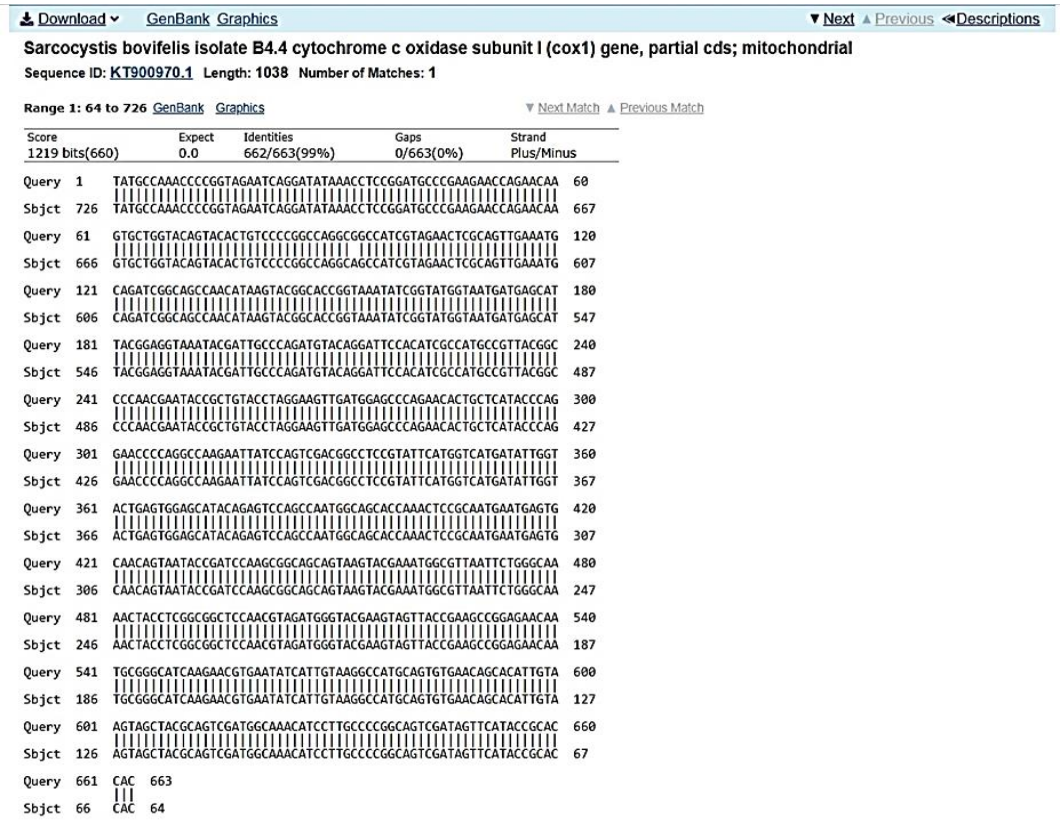


Figure 5: DNA sequence alignment of Cox 1 gene in isolated *S. bovifelis* (~ 700 bp) samples compared with the published sequences of *S. bovifelis* on GenBank (accession number: KT900970).

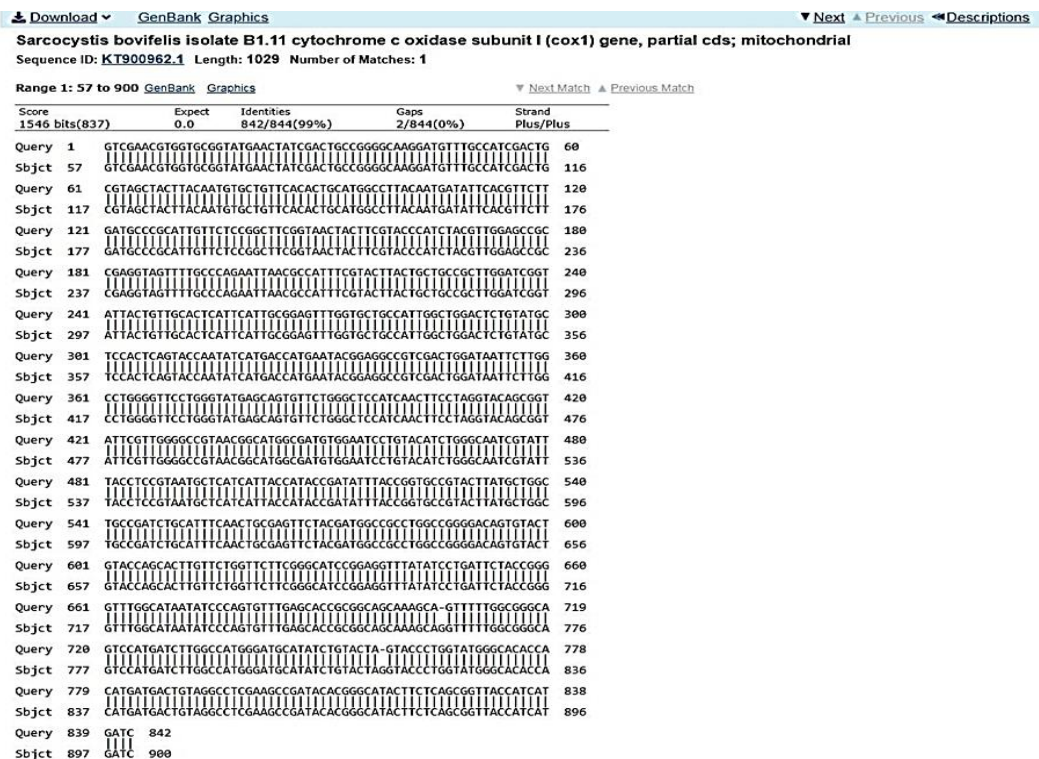


Figure 6: DNA sequence alignment of Cox 1 gene in isolated *Sarcocystis* spp. (~ 850 bp) samples compared with the published sequences of *S. bovifelis* on GenBank (accession number: KT900962).

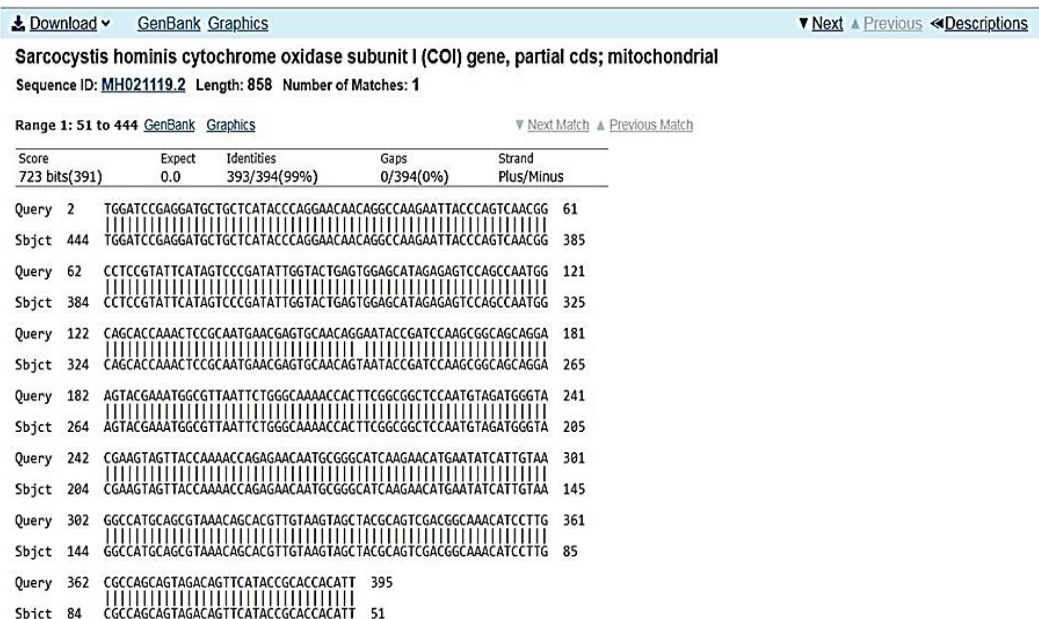


Figure 7: DNA sequence alignment of Cox 1 gene in isolated *Sarcocystis hominis* (~ 420 bp) samples compared with the published sequences of *S. hominis* on GenBank (accession number: MH021119).

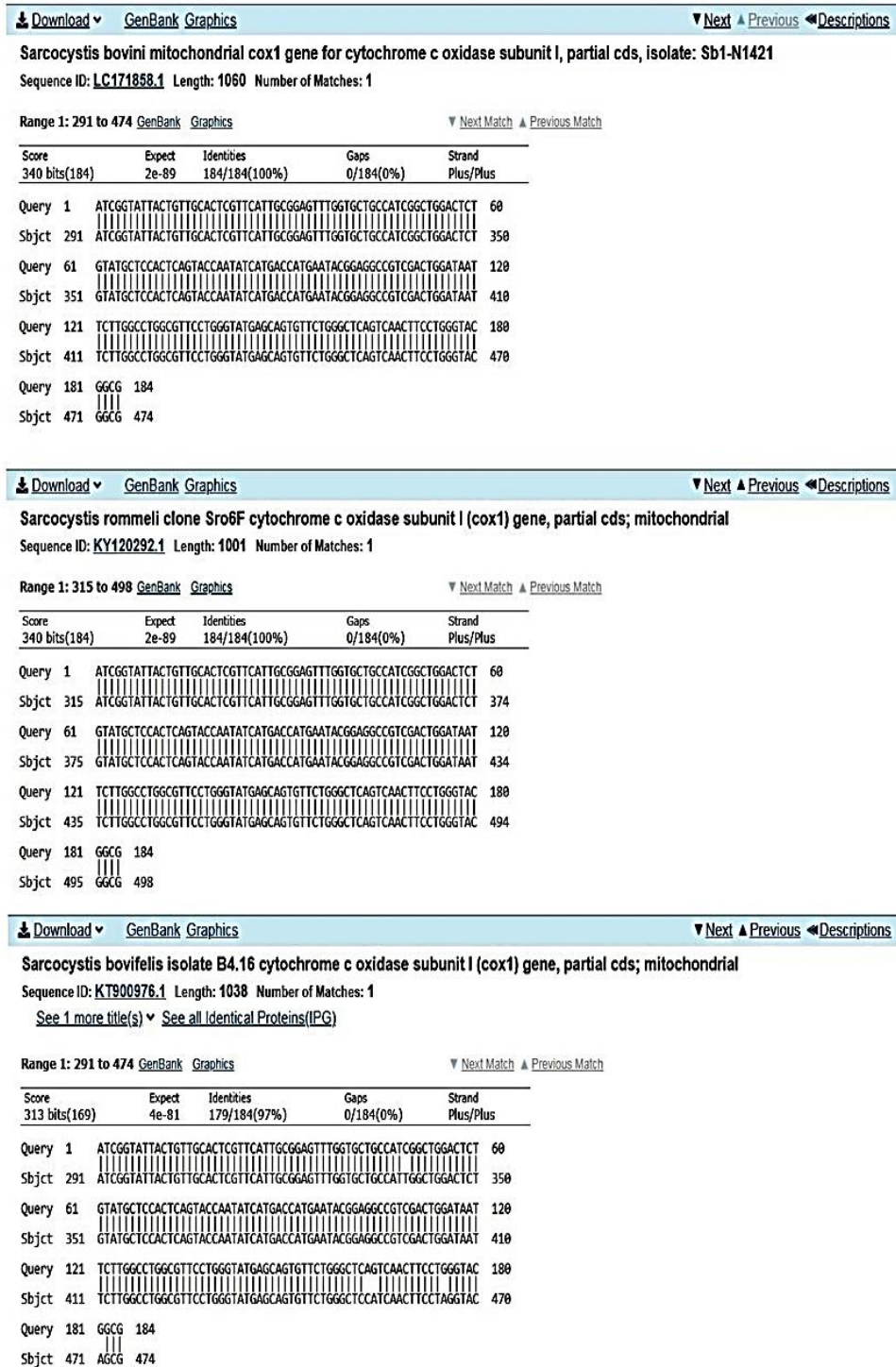


Figure 8: DNA sequence alignment of Cox 1 gene in isolated *Sarcocystis* spp. (~ 230 bp) samples compared with the published sequences of *S. bovini*, *S. rommeli* and *S. bovis* on GenBank (accession number: LC171858-KY120292-KT900976).

Phylogenetic analysis showed that the Cox 1 gene was suitable for the differentiation of *S. bovifelis* and *S. hominis* from other species (Figures 9 and 10). *Sarcocystis* spp. isolates (OK041353) shows greatest similarity with *S. bovini* and *S. rommeli* in the same topology in the phylogenetic tree. A subset was also formed with *S. bovifelis* (MT796913-MT796912) and *S. rangiferi* (KF241402-KF241401) isolates (Figure 11). According to pairwise comparisons, *S. bovifelis* and *S. hominis* showed an average genetic difference of 0.4% with other species.

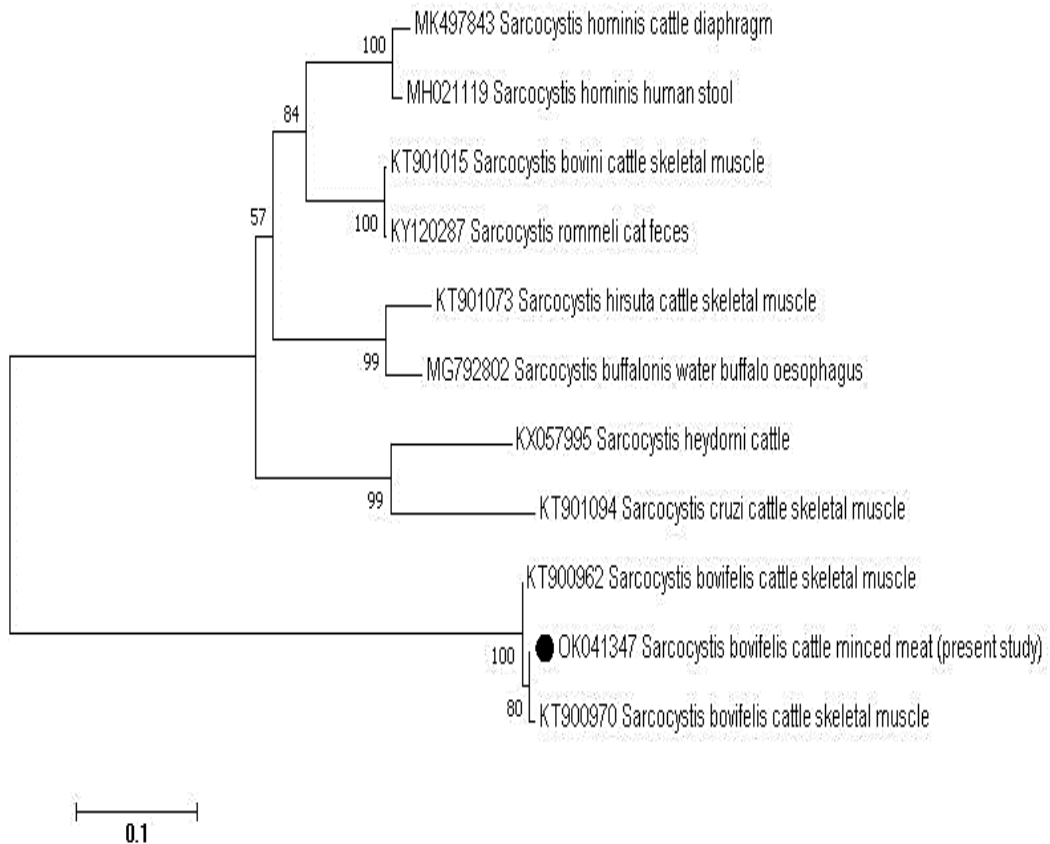


Figure 9: Neighbor-Joining phylogenetic tree of *Sarcocystis bovifelis* Cox 1 gene sequences with 2,500 bootstrap replicates. The evolutionary history was inferred by using the Kimura 2 parameter. GenBank accession numbers and sequences were obtained from the GenBank database. Isolates from this study are indicated with a black round.

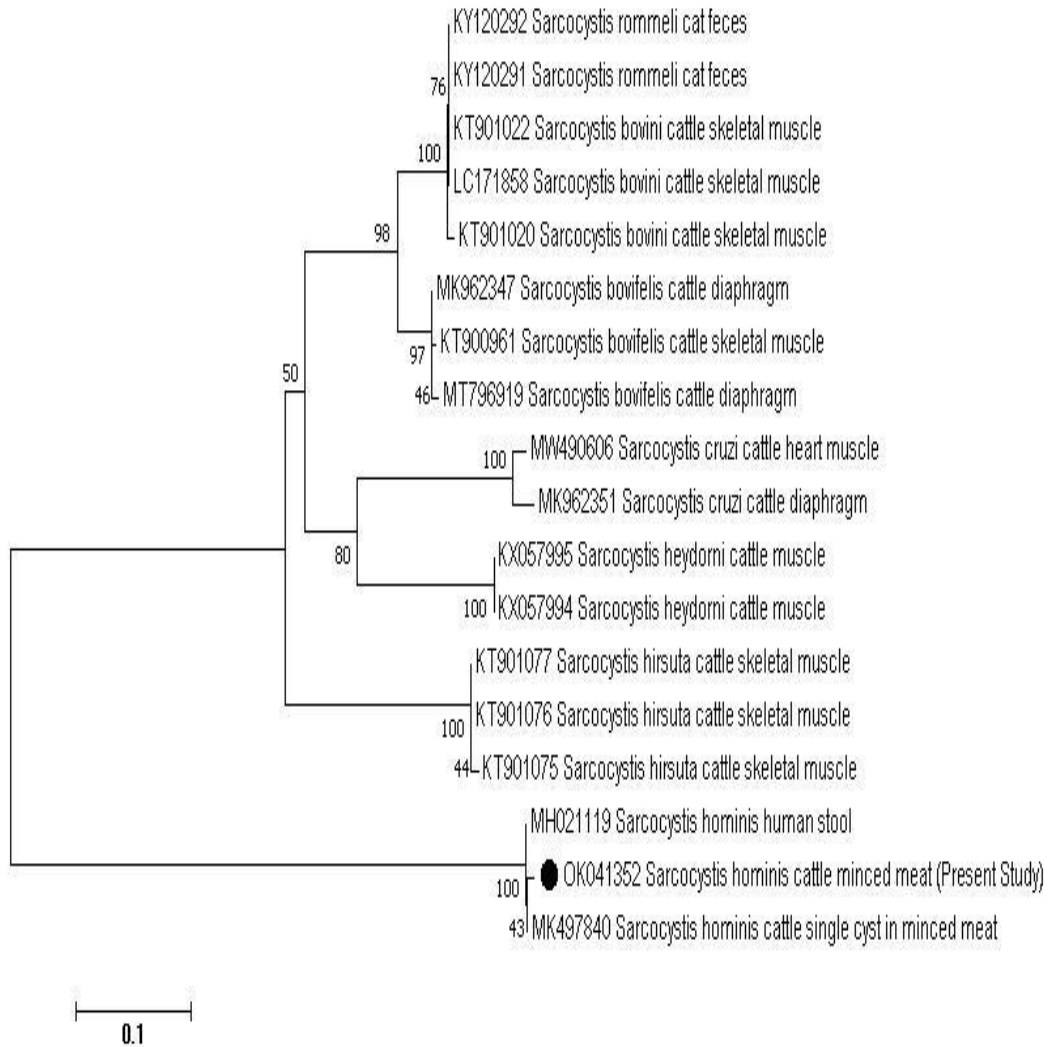


Figure 10: Neighbor-Joining phylogenetic tree of *Sarcocystis hominis* Cox 1 gene sequences with 2,500 bootstrap replicates. The evolutionary history was inferred by using the Kimura 2 parameter. GenBank accession numbers and sequences were obtained from the GenBank database. Isolates from this study are indicated with a black round.

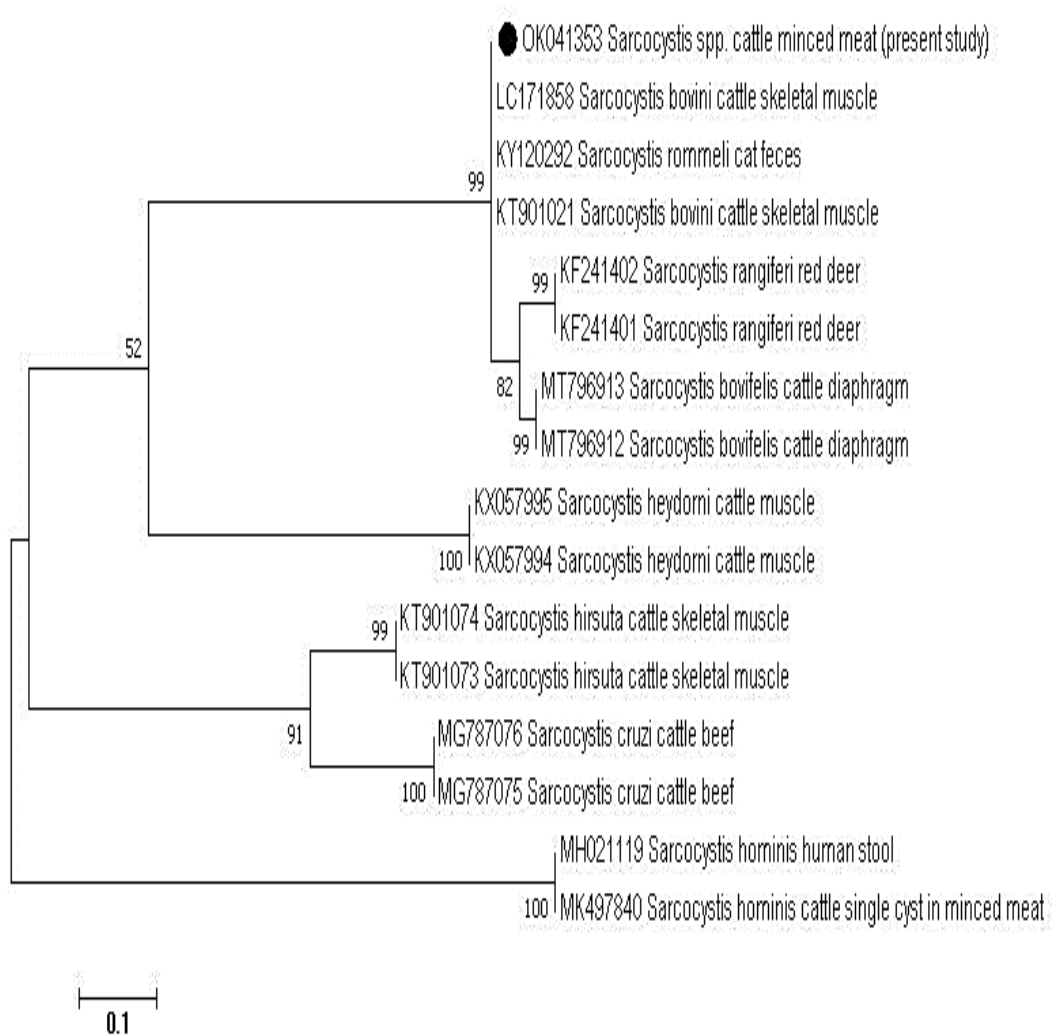


Figure 11: Maximum likelihood phylogenetic tree of *Sarcocystis* spp. Cox 1 gene sequences with 1,000 bootstrap replicates. The evolutionary history was inferred by using the Kimura 2 parameter. GenBank accession numbers and sequences were obtained from the GenBank database. Isolates from this study are indicated with a black round.

Discussion

Foods can act as carriers for disease-causing agents such as helminths, protozoa and nematodes. Biological differences in definitive hosts of *Sarcocystis* species are an important factor affecting transmission. For this reason, determining the dominant species in endemic regions is crucial in terms of ensuring effectiveness in control programs with antiparasitic drugs. The emergence of new species in cattle has led parasitologists and food safety experts to seek different methods to compare the same taxonomic groups (Gjerde, 2016). DNA sequencing and phylogenetic analyzes have been successfully applied in classification of foodborne pathogens (Gjerde, 2016; Hu et al. 2017; Rubiola et al. 2018; Prakas et al. 2020; Oğuz et al. 2021; Rubiola et al. 2021). Although the genus *Sarcocystis*

is not as outstanding as other protozoans, it is important for public health in contains zoonotic species. The identification of different species of *Sarcocystis* is great importance in terms of the epidemiology and control of sarcosporidiosis.

The 18S rRNA, cytochrome oxidase 1 (cox1) and internal transcribed spacer 1 (ITS1) genes are used in the molecular analyses of species causing cattle sarcosporidiosis. In particular, the 18S rRNA gene is more widely used for the identification of *Sarcocystis* spp. than the other genes (Gjerde, 2016; Hoeve-Bakker et al. 2019; Rubiola et al. 2019; Murata et al. 2018; Prakas et al. 2020; Oğuz et al. 2021; Rubiola et al. 2021). However, taxonomic studies have revealed that Cox 1 is the useful genetic marker for the *Sarcocystis* family (Gjerde, 2013, 2016; Rubiola et al. 2019). In a study based on the analysis of the 18S rRNA gene, the high presence of *S. hominis* in North-West Italian cattle tested by molecular methods has left some question marks in mind (Chiesa et al. 2013). Researchers confirmed the same samples with the cox 1 gene, reported that their samples were *S. bovifelis*, and therefore the role of domestic felines in bovine sarcosporidiosis should be reconsidered (Rubiola et al. 2019). In current study, *S. hominis* and *S. bovifelis* were identified. While *S. hominis* was found in one sample, it was determined that the dominant species was *S. bovifelis*. In the phylogenetic analysis of the cox1 gene; *S. bovifelis* isolates were found to be located in a single cluster on the phylogenetic tree, and it was also determined that they had a very different genetic structure from *S. hominis*, *S. bovini* and *S. rommeli*.

Prakas et al. (2020) revealed the diagnoses of *S. bovifelis*, *S. cruzi* and *S. hirsuta* with the cox1 gene. They reported that the highest overall intraspecific genetic variability was in *S. cruzi*. Measurement of intraspecific genetic variability of the species found in present study was not performed. However, overall sequence similarity was 99% compared to similar species registered in the GenBank database. Hu et al. (2017) conducted an experimental study showing that cats are definitive hosts of *S. rommeli*. They detected oocysts/sporocysts in the fecals of two cats fed bovine muscle containing *S. rommeli* cysts after a 14-15 day prepatent period. Cox1 sequences results for *S. rommelia* oocysts/sporocysts isolated from experimental fecal samples showed very high identity with those from *S. rommeli* sarcocysts (99.4%) isolated from bovine muscle tissue. However, the same samples showed similarity with *S. bovini* on average (99.5%), and the two species could not be distinguished from each other. No experimental study was conducted in current study. Primer set designed for *S. bovini* was subjected to PCR reaction and the obtained isolates showed a high rate (100%) similarity with both *S. rommeli* and *S. bovini*. On the other hand, it has been determined that it is genetically similar to *S. bovifelis* at a rate of 97%. It should be noted that *S. bovini* and *S. rommeli* have not been identified in Turkey yet. *S. bovini* has been confirmed in Argentina and New Zealand (Gjerde, 2016; Murata et al. 2018). In addition, *S. rommeli* has been found in China (Hu et al. 2017). Previous studies have been reported *S. bovifelis* in cattle from Turkey by using light microscopy (Şaki et al. 2010). These data need to be confirmed by current molecular detection techniques.

According to Cox1 phylogenetic analysis, it was determined that *S. hominis* in the same sub-genus showed high genetic differences from these three species. In previous study, we could not obtain accurate information for *S. hominis* (Oğuz et al. 2021). Because in the past study, the 18S rRNA gene region was used. This gene region is widely used for the molecular diagnosis of bovine sarcosporidiosis and has a lower discriminative. The expression *S. hominis*-like was used for unidentified samples (Rubiola et al. 2018; Oğuz et al. 2021). In the current study, the cox1 gene region and the primer set developed by Rubiola et al. (2020) were used. *S. hominis* cox1 sequence (GenBank accession number OK041352) showed 99% homology with *S. hominis* (Hoeve-Bakker et al. 2019) isolated from cattle in the Netherlands and (Rubiola et al. 2020) isolated in human feces in

Italy. In addition, according to the phylogenetic analysis results, it was revealed that *S. hominis* can be distinguished from *S. bovifelis*, *S. bovini* and *S. rommeli* in cattle due to the high level of differentiation of the mitochondrial *cox1* sequence.

Conclusion

Molecular characterizations of the species responsible for *Sarcocystis* infection were conducted in 50 DNA samples obtained from cattle minced meats in Van province, Turkey. The findings of present study, the presence of *S. bovifelis* and *S. hominis* was revealed and the primary common species was determined as *S. bovifelis*. In the present study, *S. bovifelis* and *S. hominis* were genetically characterized for the first time in cattle in the region. In Turkey, there is studies noncurrent and microscopic data of the geographical distribution of these two *Sarcocystis* species. Especially, further research on the zoonotic character of *S. hominis* is recommended.

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References

1. Chiesa F., Muratore E., Dalmaso A., Civera T. (2013). *A new molecular approach to assess the occurrence of Sarcocystis spp. in cattle and products thereof: preliminary data*. Ital. J. Food. Safety. 2: e41.
2. Dubey J.P. (2015). *Foodborne and waterborne zoonotic sarcocystosis*. Food. Waterborne. Parasitol. 1: 2–11.
3. Gjerde B. (2013). *Phylogenetic relationships among Sarcocystis species in cervids, cattle and sheep inferred from the mitochondrial cytochrome c oxidase subunit I gene*. Int. J. Parasitol. 43:579–591.
4. Gjerde B. (2016). *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. 115: 1473–1492.
5. Hall T. (1999). *BioEdit: a user-friendly biological sequence alignment editor and analysis suite*. Nucleic. Acids. Symp. Ser. 41: 95–98.
6. Hoeve-Bakker B.J.A., van der Giessen J.W.B., Franssen F.F.J. (2019). *Molecular identification targeting cox1 and 18S genes confirms the high prevalence of Sarcocystis spp. in cattle in the Netherlands*. Int. J. Parasitol. 49: 859–866.
7. Hu J.J., Huang S., Wen T., Esch G.W., Liang Y., Li H.L. (2017). *Morphology, molecular characteristics, and demonstration of a definitive host for Sarcocystis rommeli from cattle (Bos taurus) in China*. J. Parasitol. 103:471–476.
8. Kumar S., Stecher G., Tamura K. (2016). *MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets*. Mol. Biol. Evol. 33: 1870–1874.
9. Murata R., Suzuki J., Hyuga A., Shinkai T., Sadamasu K. (2018). *Molecular identification and characterization of Sarcocystis spp. in horsemeat and beef marketed in Japan*. Parasite. 25: 27.

10. Oğuz B., Değer M.S., Koşal S. (2021). *Molecular identification using 18S ribosomal RNA of Sarcocystis spp. in bovine minced meat in Van Province, Turkey*. Ankara. Univ. Vet. Fak. Derg. 68(2): 97–105.
11. Prakas P., Butkauskas D. (2012). *Protozoan parasites from genus Sarcocystis and their investigations in Lithuania*. Ekologija. 58: 45–58.
12. Prakas P., Strazdaitė-Žielenė Ž., Januškevičius V., Chiesa F., Baranauskaite A., Rudaity-Lukosiene E., Serviene E., Petkevicius S., Butkauskas D. (2020). *Molecular identification of four Sarcocystis species in cattle from Lithuania, including S. hominis, and development of a rapid molecular detection method*. Parasites. Vectors. 13: 610.
13. Rubiola S., Chiesa F., Zanet S., Civera T. (2018). *Molecular identification of Sarcocystis spp. in cattle: partial sequencing of Cytochrome C Oxidase subunit 1 (COI)*. Ital. J. Food. Saf. 7: 7725.
14. Rubiola S., Chiesa F., Zanet S., Civera T. (2019). *Molecular identification of Sarcocystis spp in cattle: partial sequencing of cytochrome c oxidase subunit 1 (COI)*. Ital. J. Food. Saf. 7: 7725.
15. Rubiola S., Civera T., Ferroglio E., Zanet S., Zaccaria T., Brossa S., Cipriani R., Chiesa F. (2020). *Molecular differentiation of cattle Sarcocystis spp. by multiplex PCR targeting 18S and COI genes following identification of Sarcocystis hominis in human stool samples*. Food. Waterborne. Parasitol. 21: 18:e00074
16. Rubiola S., Civera T., Panebianco F., Vercellino D., Chiesa F. (2021). *Molecular detection of cattle Sarcocystis spp. in North-West Italy highlights their association with bovine eosinophilic myositis*. Parasites. Vectors. 14: 223.
17. Şaki C., Değer S., Ozer E. (2010). *Türkiye’de Sarcosporidiosis*. Yüzüncü Yıl Üniversitesi Vet. Fak. Derg. (new name Van Vet J) 21 (2): 129–134. [in Turkish]