

Molecular identification using 18S ribosomal RNA of *Sarcocystis* spp. in bovine minced meat in Van Province, Turkey

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Abstract: This study aimed to determine the prevalence and molecular characterization of *Sarcocystis* spp. in bovine minced meat that is sold in various grocery stores and butcher shops in Van Province of Turkey. For this purpose a total of 150 samples were obtained from İpekyolu, Tuşba, Edremit, Erciş, and Gevaş districts of Van Province in monthly periods from May to October 2019. 28% (42/150) were found positive for *Sarcocystis* species as a result of the microscopic analyses and 96.6% (145/150) were found positive for *Sarcocystis* species as a result of the multiplex-PCR and RFLP methods. *Sarcocystis cruzi* (96.6%) was detected in all samples that were detected positive using molecular methods. *Sarcocystis hominis*-like was found in 143 (95%) samples whereas *S. hirsuta* was detected in only 4 (2.6%) samples. According to the Basic Local Alignment Search Tool (BLASTN) analysis of the 18S rRNA gene region of the *S. cruzi* (MN832695) and *S. hirsuta* (MN832697) isolates, they showed 100% similarity to the samples (MH681972; MH681973) that were submitted to GenBank from China. The BLASTN analysis of the 18S rRNA gene region of the *S. hominis*-like isolate (MN832696) revealed that it was 99.45% identical to the *S. bovini* (KT901155) isolated from a water buffalo in New Zealand. In conclusion, the molecular characterization of *Sarcocystis* spp. has been provided for the first time in Van Province, and the first unverified scientific data for *S. bovini* has been established in this study.

Keywords: 18S rRNA, bovine minced meat, PCR, *Sarcocystis* spp.

Türkiye'nin Van ilindeki sığır kıymalarında *Sarcocystis* türlerinin 18S ribosomal RNA gen bölgesi kullanılarak moleküler tanımlanması

Özet: Bu çalışmanın amacı Türkiye'nin Van ilinde çeşitli market ve kasaplarda satılan sığır kıymalarında *Sarcocystis* türlerinin yayılışını ve moleküler karakterizasyonlarını ortaya koymaktır. Bu amaçla 2019 yılının Mayıs-Ekim aylarında aylık periyotlarla Van'ın İpekyolu, Tuşba, Edremit, Erciş ve Gevaş ilçelerinden toplam 150 numune toplanmıştır. Mikroskopik analizler sonucunda %28'i (42/150) *Sarcocystis* türleri yönünden pozitif bulunmuş, multiplex-PZR ve RFLP yöntemleri sonucunda ise %96,6'sı (145/150) *Sarcocystis* türleri yönünden pozitif bulunmuştur. Moleküler yöntemlerle pozitif olarak belirlenen tüm örneklerde *S. cruzi* (%96,6) tespit edilmiştir. *Sarcocystis hominis*-like 143 (%95) örnekte bulunurken, *S. hirsuta* ise sadece 4 (%2,6) örnekte tespit edilmiştir. *Sarcocystis cruzi* (MN832695) ve *S. hirsuta* (MN832697) izolatlarının 18S rRNA gen bölgesinin Basic Local Alignment Search Tool (BLASTN) analizlerine göre, GenBank'a Çin'den kaydedilen (MH681972; MH681973) örneklerle %100 benzerlik göstermiştir. *Sarcocystis hominis*-like izolatının 18S rRNA gen bölgesinin BLASTN analizlerine göre (MN832696) Yeni Zelanda'da mandadan izole edilen *S. bovini* (KT901155) izolatı ile %99,45 identik olduğu belirlenmiştir. Sonuç olarak bu çalışma ile Van ilinde ilk kez *Sarcocystis* türlerinin moleküler karakterizasyonları sağlanmış ve *S. bovini* için doğrulanmamış ilk bilimsel veriler oluşturulmuştur.

Anahtar sözcükler: 18S rRNA, PZR, *Sarcocystis* spp., sığır kıymaları.

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 (32). *Sarcocystis* spp., heteroxen and protozoan parasites, form cysts in tissues of intermediate hosts and are thrown out as sporocysts with definitive hosts. Cysts of these species are

found in hearts, oesophagus, diaphragms, tongues, jaws and other skeletal muscles of herbivores, which are their intermediate hosts. Among herbivores, cattle are reported to be the intermediate hosts for seven species, i.e., *S. cruzi*, *S. bovifelis*, *S. hirsuta*, *S. bovini*, *S. hominis*, *S. rommeli* (formerly known as *S. sinensis*-like) and *S. heydorni*. The definitive hosts of these species are canids (*S. cruzi*), felids (*S. hirsuta*, *S. bovini*, *S. bovifelis* and *S. rommeli*) and

humans (*S. hominis* and *S. heydorni*). It has been found that among these species *S. hominis* and *S. heydorni* caused zoonotic infections, infected humans with infected pork and beef or contaminated food, and calves were infected with sporocysts excreted in feces as a result of infection (6, 10, 15, 18).

Sarcosporidiosis is generally considered non-pathogenic for cattle. Lymphadenopathy, petechial hemorrhages in serious membranes, degeneration, sarcocysts and hemorrhages in the heart, kidney, lung and muscles are detected in necropsy. Sarcocystosis in humans is found in the chronic form in the digestive system, and the symptoms are not significant or specific. Acute gastroenteritis may occur; abdominal pain and clinical symptoms such as nausea, vomiting, diarrhea can be seen. Some species in humans may cause muscular sarcocystosis, and progress to myalgia and arthralgia (8).

In 2010, the European Food Safety Authority (EFSA) highlighted the lack of reliable methods for detecting the presence of *Sarcocystis* species in animals and meat products (40). These species that cause sarcosporidiosis in cattle are generally microscopic and the size of the cysts, and the structure and thickness of the cyst walls vary according to the species. Previous studies (36, 39) conducted in the province, have identified these parasites under the light microscope which has several disadvantages. The thin cyst wall with the presence of hair-like villar protrusions of *S. cruzi* and *S. heydorni* enables them to be separated from the other two species. However, the cysts of *S. hirsuta*, *S. hominis*, *S. bovini*, and *S. rommeli* are thick-walled, and electron microscopic examination or molecular methods are required to be able to distinguish them from each other. The use of electron microscopes is strictly dependent on qualified microscopists, long working hours and demanding practices. Nevertheless, because these microscopes are expensive and not easily available, molecular applications have become more common in the diagnoses of *Sarcocystis* species (6, 8, 37).

In recent years, several molecular diagnostic techniques have been applied in the identification of *Sarcocystis* spp. in meat products (such as minced meat, meatball, raw hamburger, and sausage) that are most frequently offered for human consumption. These techniques include multiplex PCR, PCR-RFLP (Restriction Fragment Length Polymorphism), PCR-RAPD (Random Amplified Polymorphism DNA) and DNA Sequencing. The 18S rRNA, cytochrome oxidase 1 (cox1) and internal transcribed spacer 1 (ITS1) gene regions are used in the molecular analyses of species causing sarcosporidiosis. In particular, the 18S rRNA gene region is more widely used for the identification of *Sarcocystis* spp. than the other gene regions (4, 16, 18, 23, 24, 27, 33, 42). However; it has been reported that *S. hominis*, *S. bovifelis*, *S. bovini*, and *S. rommeli* species

cannot be distinguished by the 18S rRNA gene region, and the term '*S. hominis*-like' should be used for all these species (35). *Sarcocystis sinensis* is not included in this group, as it was not considered a cattle species (35).

The rate of *Sarcocystis*-infection detected in cattle tissues is considerably high (91.6-100%) in Turkey. Therefore, it is thought that Turkish people consuming raw or undercooked beef products may have serious health problems. In addition, these studies have been mostly performed using conventional diagnostic methods (2, 30, 31, 36, 39, 41), whereas there are only a limited number of molecular diagnostic studies in Turkey (12, 20). Remarkably, the molecular characterization studies are insufficient in Turkey.

The objectives of this study were to determine the prevalence of *Sarcocystis* spp. and which species are found in bovine minced meat sold in various grocery stores and butcher shops by using the molecular techniques (multiplex-PCR and RFLP) in Van Province of Turkey.

Materials and Methods

Bovine minced meat samples: In this study, 150 bovine minced meats that were purchased from various butcher shops and markets in İpekyolu, Tuşba, Edremit, Erciş and Gevaş districts of Van Province in monthly periods from May to October 2019 were used as the sample. The meats were freshly ground, (approximately 200 gr each), taken into sterile sampling bags under aseptic conditions, and brought to the laboratory in refrigerated heat-insulated containers within 30-60 minutes at the latest. The approval of the Ethics Committee for this research was obtained from the Animal Experiments Local Ethics Committee of Van Yüzüncü Yıl University (dated 28/06/2018 and numbered 06).

Isolation and microscopic examination of *Sarcocystis* spp.: Five grams of samples were taken into 50 cc trypsin solution (1.3 g pepsin, 3.5 ml 25% HCl, 2.5 g NaCl and 500 ml distilled water), kept at 40°C for 30 minutes by thawing and chopping method and homogenized through tissue homogenizer (14). Thereafter, it was strained through a 63-µm strainer, and the filtrate was placed into a tube. The homogenizer was cleaned with boiling water before each tissue. This filtrate was centrifuged at 3500 rpm for 10 minutes. Subsequently, a small quantity of sediment at the bottom was taken by a pipette and was morphologically examined by the light microscope (x40) for *Sarcocystis* tissue cysts (18, 28).

DNA extraction: The minced meats, on which thawing and chopping methods were applied, were taken into 1.5 ml microcentrifuge tubes in a 30 µl distilled water, and the DNA isolations were performed with commercial DNA extraction kit (NucleoSpin® Tissue, Macherey-Nagel, Düren, Germany) by applying the manufacturer's

tissue protocol. The DNA samples were stored at -20°C until the PCR stage. After this step, two different PCR methods (multiplex-PCR and RFLP-PCR) were applied.

Multiplex-PCR: Genomic DNAs were subjected to the multiplex-PCR by using the specific primers; F: ACAGAACCAACACGCTC and R: AACCCCTAATTC CCCGTTA (amplifying ~180 bp) for *S. hominis*-like; F: ATCAGATGAAAATCTACTACATGG and R: AACCCCTAATTC CCCGTTA (amplifying ~284 bp) for *S. cruzi* and F: CATTTCGGTGATTATTGG and R: AACCCCTAATTC CCCGTTA (amplifying ~108 bp) for *S. hirsuta* (4, 35). Five microliter of DNA sample was mixed with 12.5 μl of AmpliTaq Gold 360 Master Mix (Applied Biosystems, Life Technologies, California, USA), 0.5 μM of forward and reverse primers, and 5.5 μL of DNase- and RNase-free sterile distilled water (Biobasic, Canada), respectively, in a total volume of 25 μl . The thermal profile was set to be initial denaturation: at 95°C for 3 min; 40 cycles, denaturation: at 95°C for 60 sec, annealing at 56°C for 30 sec, extension: at 72°C for 30 sec and final extension: at 72°C for 5 min. The PCR products were loaded onto a 1.5% agarose gel, stained with ethidium bromide (0.5 mg/ml) (Sigma-Aldrich, St-Louis MO, USA) and subjected to electrophoresis at 100 volts for 2 hours (Biorad, USA). It was visualized with gel documentation system (Avegene, Taiwan).

RFLP-PCR: Genomic DNAs were subjected to the PCR by the specific primers; F: CGTGGTAATTC TATGGCTAATACA and R: TTTATGGTTAAGA CTACGACGGTA (amplifying ~900 bp) gene region from 18S rRNA for *Sarcocystis* spp. (43). Five microliter of DNA sample was mixed with 12.5 μl of AmpliTaq Gold 360 Master Mix (Applied Biosystems, Life Technologies, California, USA), 0.5 μM of forward and reverse primers, and 6.5 μL of DNase- and RNase-free sterile distilled

water (Biobasic, Canada), respectively, in a total volume of 25 μl . The thermal profile was set to be initial denaturation: at 94°C for 5 min; 35 cycles, denaturation: at 94°C for 30 sec, annealing at 58°C for 1 min, extension: at 72°C for 1 min and final extension: at 72°C for 5 min. The PCR products were subjected to 1.5% agarose gel electrophoresis (90 volts for 60 min), visualized and analyzed by gel documentation system (Avegene, Taiwan).

The amplified PCR products were digested with BcII restriction endonuclease enzyme (ER0721, Thermo Fisher Scientific, USA) and different *Sarcocystis* spp. were detected. Fragments of ~358 and 595 bp lengths for *S. hirsuta* and ~782 and 140 bp lengths for *S. hominis*-like were expected whereas it was expected to be single band (i.e. uncut) for *S. cruzi* (16). For RFLP analysis; 8 μl PCR product, 5 μl distilled water, 1 μl restriction enzyme and 1 μl buffer were added into a total of 15 μl reaction mixture, and incubated at 55°C for 3 h. After that the enzyme was inactivated at 80°C for 20 min. The fragments (10 μl) were detected on 1.5% agarose gel electrophoresis (90 volts for 60 min) and visualized by Gel Documentation System or UV Transilluminator Equipment.

Sanger sequencing: Following the multiplex and RFLP-PCR stages, expected bands were determined, and the PCR process was applied again to form a single band for each parasite by switching the conventional PCR (Figure 1). The PCR products and primers coding the 18S rRNA gene region were properly packaged and sent to the Sentebiolab Company (Ankara) for performing DNA sequence analysis. The sequence chromatograms were controlled and arranged using the BioEdit software (13). The final consensus sequences of our isolates were subjected to the "BLAST analysis" (<http://www.ncbi.nlm.nih.gov/BLAST>) in the GenBank

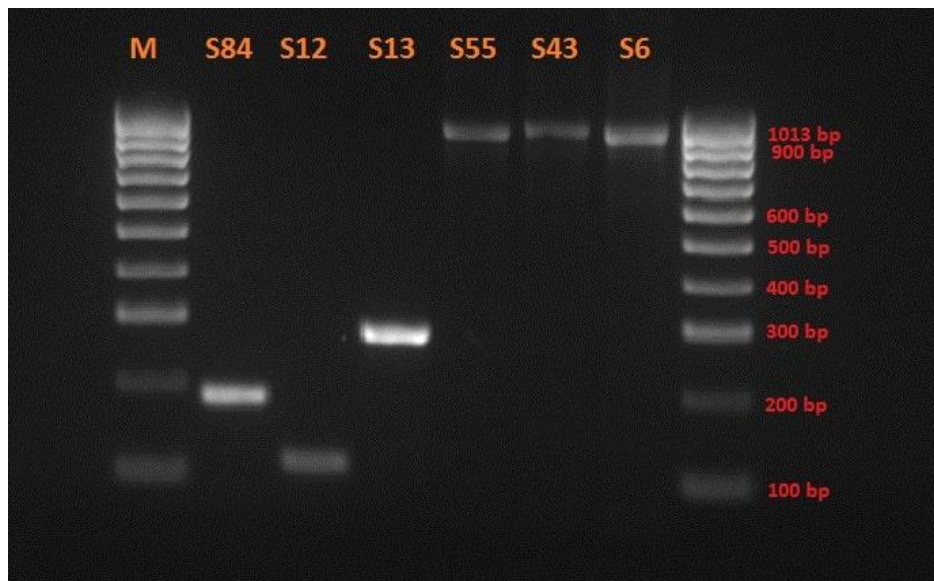


Figure 1. The single-PCR products of *Sarcocystis* species. M: 100 bp molecular size marker, S84: *S. hominis*-like (~180 bp), S12: *S. hirsuta* (~108 bp), S13: *S. cruzi* (~284 bp). S55, S43 and S6: *Sarcocystis* spp. (~900 bp).

Database, 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 (21). The 18S rRNA phylogenetic analysis data set was comprised of nucleotide sequences of a total of 23 isolates. *Hepatozoon canis* (MH615005) was used as an “outgroup”. The phylogenetic analysis and the construction of phylogenetic tree were performed with 1000-repeated bootstrap using “maximum likelihood (ML)” method in the MEGA 7.0 (21) software. The nucleotide sequences obtained in the study were submitted as the corresponding accession numbers of MN832695-MN832699 in GenBank.

Results

The microscopic analysis revealed that 42 (28%) of 150 minced meat samples were positive for *Sarcocystis* microcyst (Figure 2). As a result of the multiplex-PCR analysis, 145 (96.6%) of 150 minced meat samples were found positive for *Sarcocystis* species. *Sarcocystis cruzi* was determined in all (96.6%) positive samples. Moreover, *S. hominis*-like was found in 143 (95%) samples, whereas *S. hirsuta* was detected in only 4 (2.6%) samples. Expected fragments of ~284 bp for *S. cruzi*, ~180 bp for *S. hominis*-like and ~108 bp for *S. hirsuta* were successfully amplified (Figure 3). In the RFLP-PCR stage, bands of ~900 bp were first expected and successfully

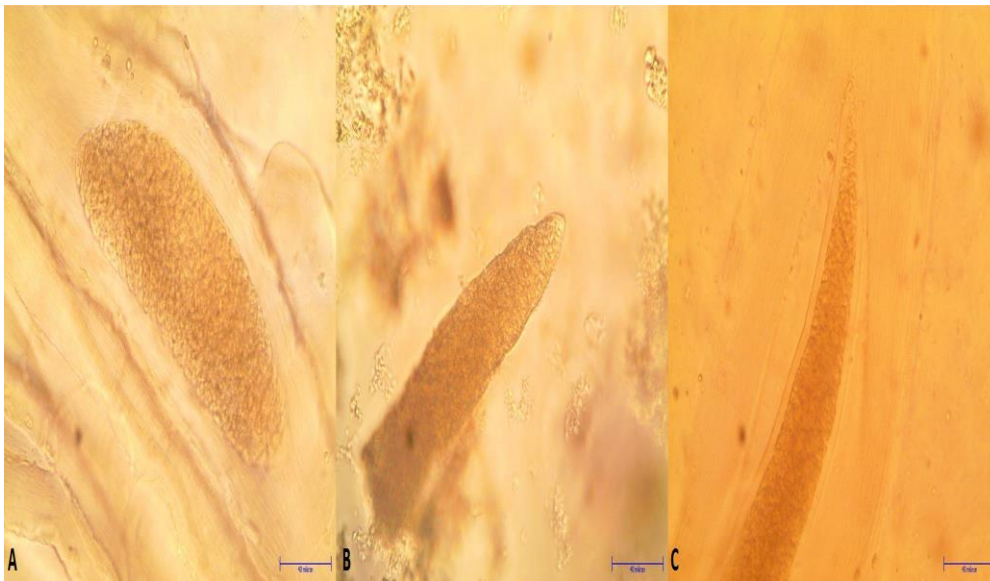


Figure 2. Microscopic determination of *Sarcocystis* microcyst. A: *S. cruzi*, B: *S. hirsuta*, C: *S. hominis*-like (*S. bovis* (unascertained information)), Scale bars: 40 µm.

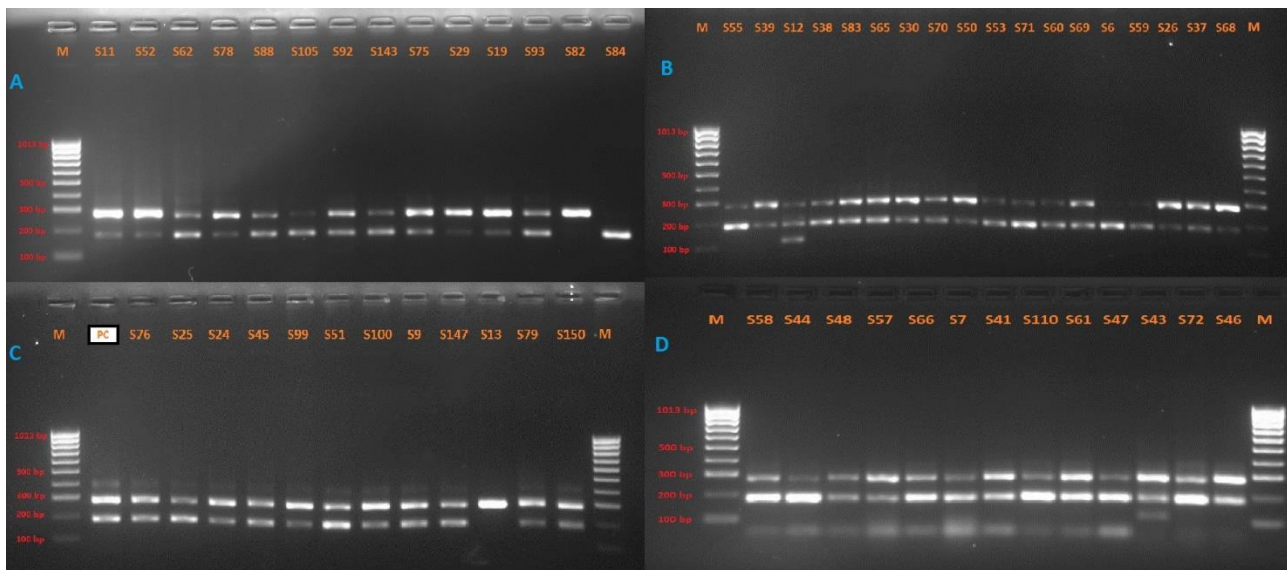


Figure 3. The analysis of multiplex-PCR products. M: 100 bp molecular size marker; PC (C): positive mPCR controls, S11(A), S55(B), S76(C), S58 (D): *S. cruzi*/*S. hominis*-like, S82(A) and S13(C): *S. cruzi*, S84(A): *S. hominis*-like, S12(B) and S43(D): *S. cruzi*/*S. hominis*-like/*S. hirsuta*.

amplified (Figure 4). According to PCR-RFLP analysis of the species identification of *Sarcocystis*, the digested 900 bp amplified fragments using BclI revealed that 145 (96.6%) of the 150 isolates were *S. cruzi*, 4 (2.6%) showed co-infection to *S. cruzi* and *S. hirsuta*, 143 (95%) was mixed infected to *S. cruzi* and *S. hominis-like*, 4 (2.6%) showed the pattern of mix infection to three species (Figure 5). The RFLP-PCR results were found to be compatible with the molecular prevalence rate (96.6%) as compared with the multiplex-PCR results.

Our *S. cruzi* (MN832695) isolates was found to be 100% similar to (MH681972) from China. In addition, the microcyst of *S. cruzi* obtained in the study of Imre et al. (18) was found to have similar morphological features with the microcyst taken in our study (Figure 1/A). Similarly, our *S. hirsuta* (MN832697) isolates showed 100% identity with the isolate (MH681973) from China. It is noteworthy that the microscopic photography of *S. hirsuta* obtained in the study of Murata et al. (28) was similar to the one in our study (Figure 1/B).

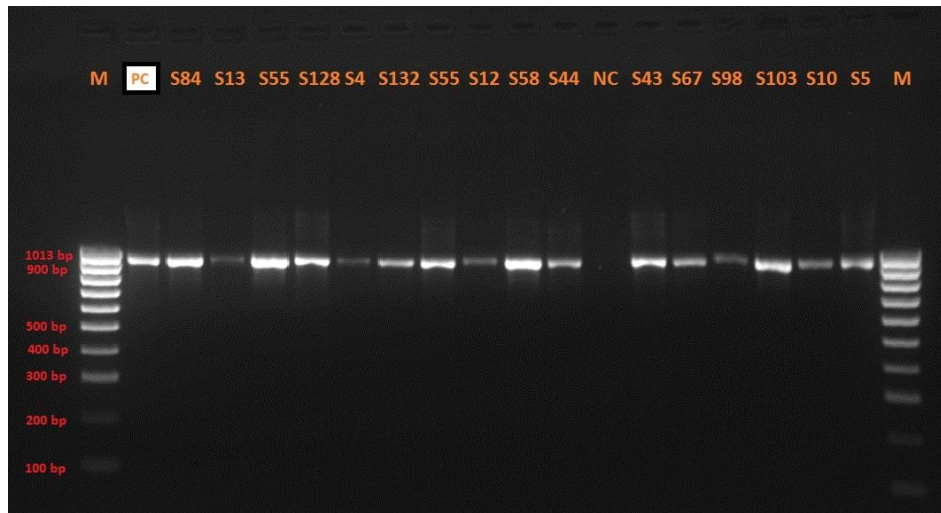


Figure 4. The single-PCR products of *Sarcocystis* species. M: 100 bp molecular size marker, PC: Positive PCR controls, NC: Negative PCR control, S84-S44/S43-S5: *Sarcocystis* spp. positives (~900 bp).

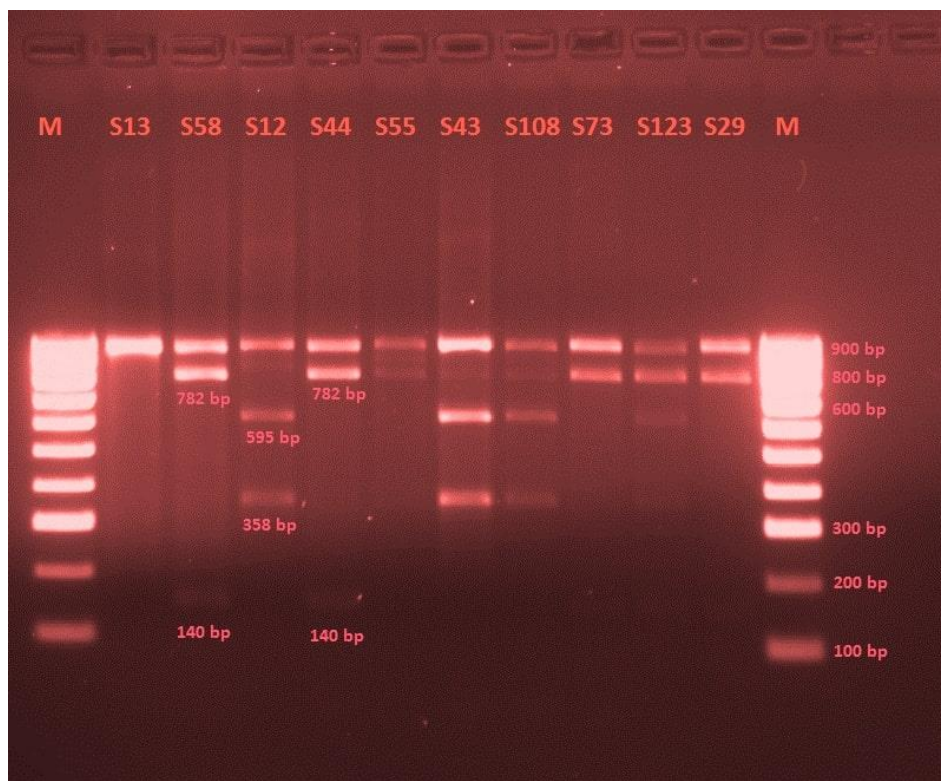


Figure 5. The BclI restriction endonuclease reaction of *Sarcocystis* spp. PCR products. M: 100 bp molecular size marker, S13: *S. cruzi*, S58-S44: *S. cruzi* mixed with *S. hominis-like*, S12: *S. cruzi* mixed with *S. hirsuta*.

The nucleotide percent identities among 18S rRNA sequence of *S. hominis*-like from Turkey (MN832696) showed 99.45% identity with *S. bovini* (KT901155) from New Zealand. Furthermore, our *S. hominis*-like from Turkey (MN832696) differed by one nucleotide (A to G) in the 18S rRNA sequence of *S. bovini* (KT901155) from New Zealand (Figure 6). Additionally, it is worth noting

that the microscopic view of *S. bovini* obtained in the study of Murata et al. (28) was similar to the microcyst found in our study (Figure 1/C). Figure 7 shows the phylogenetic tree constructed with ML method (Kimura 2 parameter) of *Sarcocystis* isolates identified from minced meat in Van Province and some other *Sarcocystis* isolates from various parts of the world.

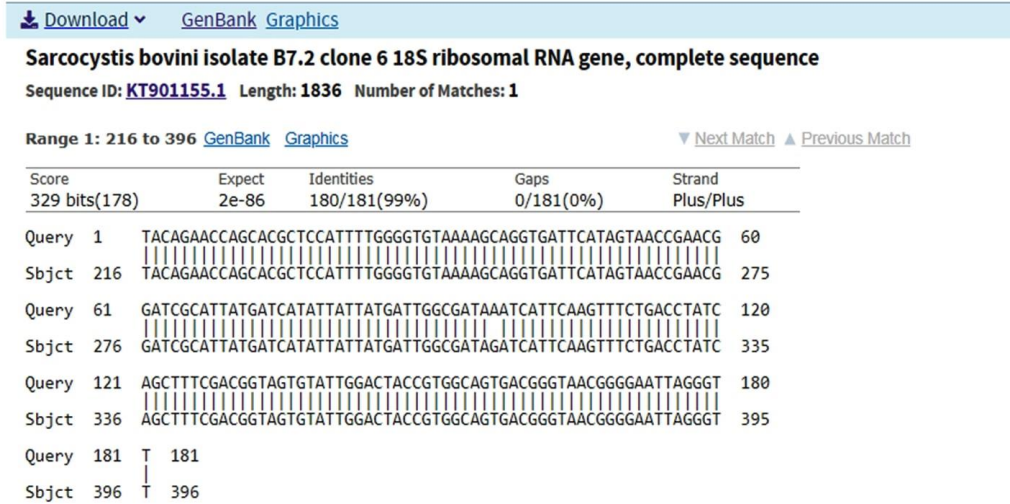


Figure 6. DNA sequence alignment of 18S rRNA gene in isolated *S. hominis*-like samples compared with the published sequences of *S. bovini* on GenBank (Accession no: KT901155).

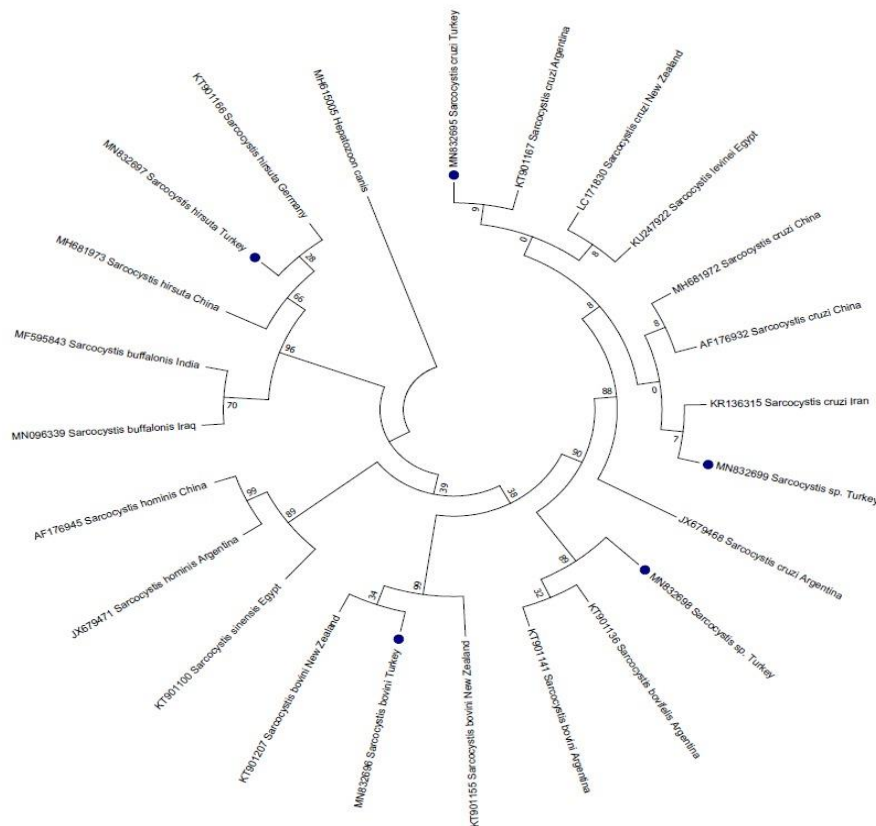


Figure 7. Maximum likelihood phylogenetic tree of *Sarcocystis* spp. 18S rRNA gene sequences with 1,000 bootstrap replicates. The evolutionary history was inferred by using the Kimura 2 parameter. Sequences were obtained from the GenBank database and GenBank accession numbers and country of origin from which the sequences were derived are included for each sequence. Isolates from this study are indicated with a blue round.

Discussion and Conclusion

Cattle are the most common intermediate host of seven *Sarcocystis* species, i.e., *S. cruzi*, *S. bovifelis*, *S. hirsuta*, *S. bovini*, *S. hominis*, *S. rommeli* (formerly known as *S. sinensis*-like) and *S. heydorni*, in their muscles (6, 8, 15, 18, 28, 35).

The results of the prevalence of *Sarcocystis* spp. infection reported in this study are consistent with the ones found in similar studies. The prevalence of infection in cattle in Turkey and other countries around the world has been reported to be high. For example, a 100% prevalence of infection in cattle has been reported in countries such as Brazil (38), Iran (29), the USA and Argentina (25). Studies conducted on *Sarcocystis* spp. in cattle in different parts of Turkey using the microscopic methods reported high prevalence rates of 100% in Bayburt region (30), 91.5% in Elazığ region (31), 100% in Bursa region (41), 92% in Van region (39), 95.3% in Kars region and 97.1% in Erzurum region (2). The prevalence of infected cattle varies between 66.0% and 78.1% in Hungary (17), 57.5% and 83.6% in Estonia (22), 78.1% and 91% in Italy (4, 5), 64.6% and 100% in Portugal (11), and 90% and 100% in France (3). Lower prevalence of infected cattle, were determined in Germany and Romania, i.e., 26.4% and 17.9%, respectively (26). To sum up, it is difficult to carry out a detailed analysis and comparison of the data on prevalence of *Sarcocystis* spp. infection in domestic animals. For example, in a study in Romania, the researchers only performed the PCR analyses of the samples that they found to be positive under a microscope. If they had examined all the samples with the PCR method, the rate they detected would have been expected to be even greater (18). In other words, the methods used in *Sarcocystis* infections can change the prevalence rates considerably. The results of this study also prove this fact. It has also been reported that the prevalence of infection can vary significantly depending on the muscle groups. In a study on *Sarcocystis* in different muscle groups in cattle, the prevalence of infection was found to be 98.1% in oesophagus, while it was 44.9% in lumbar muscles (*M. longissimus dorsi*) (19). Considering the public health, the accurate data on the prevalence of *S. hominis* in cattle is needed, and nowadays reliable and fast molecular methods for the identification are preferred.

Techniques based on DNA sequence analysis have been successfully applied for the classification of foodborne pathogens, improving diagnostic methods and phylogenetic analyses (4, 10, 18, 23, 28, 35). In the current research, *Sarcocystis* spp. isolated from cattle was attempted to identify by using 18S rRNA gene region. The rate of *Sarcocystis* in minced meat was determined as 96.6% by applying two different molecular techniques. The prevalence rate of *Sarcocystis* was found to be close to the ones found in the studies previously conducted in

Turkey (2, 30, 31, 36, 39). The primers used in the multiplex PCR analysis were taken from Chiesa et al. (4). They also reported in a different study they conducted five years later that the primer of *S. hominis* they synthesized was required to evaluate as *S. hominis*-like (*S. hominis*, *S. bovifelis*, *S. bovini* and *S. rommeli*) and these species could not be clearly distinguished by 18S rRNA analysis (35). *Sarcocystis cruzi* was found in all (96.6%) positive samples in the evaluation of our study results. *Sarcocystis hominis*-like was found in 143 (95%) samples whereas *S. hirsuta* was detected in only 4 (2.6%) samples.

Sarcocystis cruzi is the most commonly found species in *Sarcocystis* spp. that is found in cattle in the whole world and Turkey, as well (18, 23, 39). In this study, it was found to be 96.6% using the molecular methods. From an epidemiological point of view, infected carnivores are considered to shed *S. cruzi* sporocysts onto the nature at very high levels. These sporocysts can maintain their viability for long periods for various reasons (such as high temperatures or freezing). Pasture cattle can easily ingest these contaminated feces shedding to the environment. Sarcosporidiosis caused by *S. cruzi* in cattle is very difficult to diagnose in living animals. Several studies claim that eosinophilic myositis and sudden deaths in cattle are associated with *S. cruzi* (1, 9, 17). Microscopic or molecular methods are used in postmortem diagnosis of *Sarcocystis* species. Moussa et al. (27) compared the macroscopic, microscopic and molecular methods in a study conducted on 55 imported frozen buffalo meat in Alexandria, Egypt. In the examined samples, *Sarcocystis* spp. infection was found to be 23% macroscopically, 20% microscopically and 70.9% by molecular methods. The molecular analyses of cysts determined macroscopically and microscopically showed that the imported buffalo meat was infected with *S. fusiformis* and *S. cruzi* species, respectively. In a study conducted by Pritt et al. (33) in the USA, the histological and PCR methods were compared for the detection of *Sarcocystis* spp. on 110 beef meat samples. The PCR method was reported to be more sensitive than histological studies and gave an accurate result for species identification. In addition, in the sequence analysis result of the positive samples, *S. cruzi* was determined in all samples while *S. hominis* could not. In the study conducted on the hearts and oesophagus of cattle in Egypt, El-Kady et al. (7) reported that *Sarcocystis* spp., which were morphologically similar, were two different species (*S. cruzi* and *S. hjorti*) as a result of genetic analyses, and these species did not have host specificity. In this study, 18S rRNA gene region was obtained for *S. cruzi* in minced meat for human consumption. According to the *Sarcocystis* morphology, the sequences of the 18S rRNA gene region of the isolate identified as *S. cruzi* were verified on the basis of species as a result of BLASTN

analyses performed with *Sarcocystis* isolates submitted to GenBank database from different countries. The results of the analyses showed that our isolate named TRVBO1 (MN832695) was found to be 100% similar to *S. cruzi* isolate (MH681972) found in cattle in China.

In the current study, *S. hirsuta* was identified in only 4 (2.6%) samples. Murata et al. (28) isolated this species in one sample in Turkey. In the study conducted on 200 raw hamburgers in Kashan, Iran, Hooshyar et al. (16) found *S. hirsuta* in only two samples. The reason for the low prevalence rate in these studies can be attributed to lower frequency of encounters between cats and cattle. In addition, as a result of the phylogenetic analyses, *S. hirsuta* (MH681973) isolate obtained from China showed 100% similarity with our isolate (MN832697).

One of the positive samples determined as *S. hominis*-like was sent for sequence analysis. The sequence result of *S. hominis*-like (MH832696) was subjected to comparative analysis in the GenBank database and found to be 99.45% similar to *S. bovis* (KT901155) isolated from New Zealand. The genetic distance between the isolates was calculated at a rate of 0.011%. This finding constitutes the first scientific report for Turkey. However, recent studies confirm that the *cox1* mitochondrial gene has a higher differential power for *Sarcocystis* species. Given the taxonomic confusion between *Sarcocystis* species, it is also reported that identification of a gene that can be used as a reference gene is very important for the clear identification of *Sarcocystis* species, which are very close phylogenetically, is of great importance (15, 28, 34, 35). Therefore, we believe that it would not be accurate to make a definitive diagnosis for our isolate without *cox1* gene verification. For this reason, there is no definite finding for zoonotic *S. hominis* in minced meat in Van Province, Turkey. In fact, from a broad perspective, there is no substantial finding for Turkey. The studies date back to old times and most of them constitute microscopic data. In the future we think that it would be appropriate to carry out more detailed studies covering different gene regions for both *S. hominis* and *S. bovis*. In addition, in our bordering neighbour Iran, *S. hominis* was detected in a molecular study on raw hamburgers (24).

In conclusion, this is the first study combining classical morphological methods and advanced molecular diagnostic techniques on *Sarcocystis* species in minced meat in Van Province, and the molecular characterization of *S. cruzi* and *S. hirsuta* has been introduced for the first time in Turkey. The first data offering an insight into further studies on *S. bovis* have been also established in this study. In addition, species identified as *S. hominis*-like and *S. sinensis*-like need to be distinguished or verified with gene regions with high interspecific and low intraspecific variation. As expected, *Sarcocystis* species were found in high prevalence in minced meat. Although

there is no definite finding on *S. hominis* related to public health in our study, it is recommended that meat and meat products to be consumed be frozen in deep freeze for 1 day or cooked over 70°C for 8-15 minutes (20, 37). Additionally, it is very important to control the disease in that the organs and tissues of the intermediate host should not be fed to cats or dogs raw or undercooked.

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Ethical Statement

This study was approved by the Van Yüzüncü Yıl University Animal Experiments Local Ethics Committee (dated 28/06/2018 and numbered 06).

Conflict of Interest

The authors declared that there is no conflict of interest.

References

1. Araoz V, da Silva Silveira C, More G, et al (2019): *Fatal Sarcocystis cruzi*-induced eosinophilic myocarditis in a heifer in Uruguay. *J Vet Diagn Invest*, **31**, 656-660.
2. Arslan MÖ, Umur Ş (1997): *The prevalence of sarcocystiosis in cattle in Kars and Erzurum Province, Türkiye*. *Türkiye Parazitoloj Derg*, **21**, 417-420.
3. Bertin M, Lemieux D, Rossero A, et al (2014): *Sarcocystis hominis* is frequently associated with bovine eosinophilic myositis. *Renc Rech Ruminants*, **21**, 321-324.
4. Chiesa F, Muratore E, Dalmasso A, et al (2013): *A new molecular approach to assess the occurrence of Sarcocystis spp. in cattle and products thereof: preliminary data*. *Ital J Food Saf*, **2**, e41.
5. Domenis L, Peletto S, Sacchi L, et al (2011): *Detection of a morphogenetically novel Sarcocystis hominis-like in the context of a prevalence study in semi-intensively bred cattle in Italy*. *Parasitol Res*, **109**, 1677-1687.
6. Dubey JP (2015): *Foodborne and waterborne zoonotic sarcocystosis*. *Food Waterborne Parasitol*, **1**, 2-11.
7. El-Kady AM, Hussein NM, Hassan AA (2018): *First molecular characterization of Sarcocystis spp. in cattle in Qena Governorate, Upper Egypt*. *J Parasit Dis*, **42**, 114-121.
8. Fayer R, Esposito DH, Dubey JP (2015): *Human infections with Sarcocystis species*. *Clin Microbiol Rev*, **28**, 295-311.
9. Freiler PF, Mayhew IG, Pollock R (1979): *Bovine sarcocystosis: pathologic features of naturally occurring infection with Sarcocystis cruzi*. *Am J Vet Res*, **40**, 651-657.
10. Gjerde B (2016): *Molecular characterisation of Sarcocystis bovifelis, Sarcocystis bovis n. sp., Sarcocystis hirsuta and Sarcocystis cruzi from cattle (Bos taurus) and Sarcocystis sinensis from water buffaloes (Bubalus bubalis)*. *Parasitol Res*, **115**, 1473-1492.

11. **Goncalo JA** (2014): *Muscle parasites by Sarcocystis spp. and Cysticercus bovis (Taenia saginata) in cattle of the Azores (In Portuguese)*. PhD Thesis. University of Lisbon, Lisbon, Portugal.
12. **Güçlü F, Aldemir OS, Güler L** (2004): *Differential identification of cattle Sarcocystis spp. by random amplified Polymorphic DNA-Polymerase chain reaction (RAPD-PCR)*. Revue Med Vet, **155**, 440-444.
13. **Hall TA** (1999): *BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT*. Nucleic Acids Symp Ser, **41**, 95-98.
14. **Hamidinejat H, Razi Jalali MH, Nabavi L** (2010): *Survey on Sarcocystis Infection in Slaughtered Cattle in South-West of Iran, Emphasized on Evaluation of Muscle Squash in Comparison with Digestion Method*. J Animal Vet Adv, **9**, 1724-1726.
15. **Hoeve-Bakker BJA, van der Giessen JWB, Franssen FFJ** (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.
16. **Hooshyar H, Abbaszadeh Z, Sharafati-Chaleshtori R, et al** (2017): *Molecular identification of Sarcocystis species in raw hamburgers using PCR-RFLP method in Kashan, central Iran*. J Parasit Dis, **41**, 1001-1005.
17. **Hornok S, Mester A, Takacs N, et al** (2015): *Sarcocystis-infection of cattle in Hungary*. Parasit Vectors, **8**, 69.
18. **Imre K, Dărăbuș G, Tîrziu E, et al** (2019): *Sarcocystis spp. in Romanian slaughtered cattle: Molecular characterization and epidemiological significance of the findings*. Biomed Res Int, Article ID 4123154.
19. **Januskevicius V, Januskeviciene G, Prakas P, et al** (2019): *Prevalence and intensity of Sarcocystis spp. infection in animals slaughtered for food in Lithuania*. Vet Med (Praha), **64**, 149-157.
20. **Kamber U, Arslan MÖ, Gülbaz G, et al** (2018): *Identification of Sarcocystis spp. by polymerase chain reaction and microscopic examination in various beef products (minced meat, meatballs, fermented sausage)*. Turk J Vet Anim Sci, **42**, 1-6.
21. **Kumar S, Stecher G, Tamura K** (2016): *MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets*. Mol Biol Evol, **33**, 1870-1874.
22. **Lassen B, Talvik H** (2009): *Parasitic protozoans in livestock and pets in Estonia*. Review. Vet Zootech-Lith (Vet Med Zoot), **46**, 30-36.
23. **Meistro S, Peletto, S, Pezzolato M, et al** (2015): *Sarcocystis spp. prevalence in bovine minced meat: a histological and molecular study*. Ital J Food Saf, **4**, 4626.
24. **Moghaddam Ahmadi M, Hajimohammadi B, Eslami G** (2015): *First identification of Sarcocystis hominis in Iranian traditional hamburger*. J Parasit Dis, **39**, 770-772.
25. **More G, Abrahamovich P, Jurado S, et al** (2011): *Prevalence of Sarcocystis spp. in Argentinean Cattle*. Vet Parasitol, **177**, 162-165.
26. **More G, Pantchev A, Skuballa J, et al** (2014): *Sarcocystis sinensis is the most prevalent thick-walled Sarcocystis species in beef on sale for consumers in Germany*. Parasitol Res, **113**, 2223-2230.
27. **Moussa MM, Hiekal FA, El-Hoshy SM, et al** (2016): *Characterization of Sarcocystis species based on traditional and molecular methods in imported frozen buffalo meat in Egypt*. Alexandria J Vet Sci, **51**, 155-161.
28. **Murata R, Suzuki J, Hyuga A, et al** (2018): *Molecular identification and characterization of Sarcocystis spp. in horsemeat and beef marketed in Japan*. Parasite, **25**, 27.
29. **Nourollahi-Fard SR, Asghari M, Nouri F** (2009): *Survey of Sarcocystis infection in slaughtered cattle in Kerman, Iran*. Trop Anim Health Prod, **41**, 1633-1636.
30. **Okur H, Kandemir O, Şahin I** (1995): *An investigation on Sarcocystis species in the cattle and the sheep in Bayburt*. T Parazitol Derg, **19**, 113-118.
31. **Özer E** (1988): *Elazığ mezbahasında kesilen sığır ve mandalarda Sarcocystis türleri ve insidensi üzerinde araştırmalar*. TU Vet Hay Derg, **12**, 130-139.
32. **Prakas P, Butkauskas D** (2012): *Protozoan parasites from genus Sarcocystis and their investigations in Lithuania*. Ekologija, **58**, 45-58.
33. **Pritt B, Trainer T, Simmons-Arnold L, et al** (2008): *Detection of Sarcocystis parasites in retail beef: A regional survey combining histological and genetic detection methods*. J Food Prot, **71**, 2144-2147.
34. **Ren M, Wu F, Zou Y, et al** (2019): *Molecular characterization of Sarcocystis species isolated from Chinese buffaloes in Guizhou province based on 18S rRNA and cox1 sequences*. Mitochondrial DNA Part B, **4**, 637-641.
35. **Rubiola S, Chiesa F, Zanet S, et al** (2018): *Molecular identification of Sarcocystis spp. in cattle: Partial sequencing of cytochrome c oxidase subunit I (COI)*. Ital J Food Saf, **7**, 7725.
36. **Sancak YC, Akdemir C, Berktaş M** (1995): *Van piyasasında tüketime sunulan et ve et ürünlerinde sarcosporidiosis'in yayılışı*. Van Sağ Bil Derg, **2**, 114-116.
37. **Sevinç F** (2013): *Sığırlarda Sarcosporidiosis*. 75-82. In: MA Özcel (Ed), Veteriner Hekimliğinde Parazit Hastalıkları. Türkiye Parazitoloji Derneği, İzmir.
38. **Silva NRS, Rodrigues RJD, Araujo FAP, et al** (2002): *Detection of bovine Sarcocystis cruzi cysts in cardiac muscles: A new technique of concentration for diagnostic*. Acta Sci Vet, **30**, 127-129.
39. **Taşçı S, Toparlak M, Gül Y** (1989): *Van mezbahasında kesilen sığırlarda sarcosporidiosis'in yayılışı*. Ankara Univ Vet Fak, **36**, 254-259.
40. **Taylor MA, Boes J, Boireau P, et al** (2010): *Developent of harmonised schemes for the monitoring and reporting of Sarcocystis in animals and foodstuffs in the European Union*. Available at <http://www.efsa.europa.eu/en/supporting/pub/33e.htm>. (Accessed May 12, 2020).
41. **Tüzer E, Demir S, Aydın O** (1987): *Bursa yöresi sığırlarında sarcosporidiosis*. İstanbul Üniv Vet Fak Derg, **13**, 21-30.
42. **Vangeel L, Houf K, Chiers K, et al** (2007): *Molecular based identification of sarcocystis hominis in Belgian minced beef*. J Food Prot, **70**, 1523-1526.
43. **Yang ZQ, Li QQ, Zuo YX, et al** (2002): *Characterization of Sarcocystis species in domestic animals using a PCR-RFLP analysis of variation in the 18S rRNA gene: A cost effective and simple technique for routine species identification*. Exp Parasitol, **102**, 212-217.