Parasitol Res (2011) 108:949–954
DOI 10.1007/s00436-010-2137-y

ORIGINAL PAPER

Ultrastructural and molecular characterization of *Sarcocystis* isolated from camel (*Camelus dromedarius*) in Iran

Gholam Reza Motamedi · Abdolhossein Dalimi · Abas Nouri · Khosro Aghaeipour

Received: 9 September 2010 / Accepted: 15 October 2010 / Published online: 9 November 2010

Abstract *Sarcocystis cameli* was first described in one-humped camels (*Camelus dromedarius*), and it is the only species which have so far reported in camels. Although more than 150 species of *Sarcocystis* were described in various animals, only a few data on camel *Sarcocystis* ultrastructure were published, and this report is the first for molecular information (DNA sequence and RLFP digestion pattern). The main objective of the present work is to characterize *Sarcocystis* isolated from camels by electron microscopy and PCR-RFLP methods. Muscle samples were taken from the fresh esophagus, diaphragm, skeletal muscles, and heart of one-humped camels (*C. dromedarius*) slaughtered in abattoirs of Tehran and Ghazvin provinces, Iran. The dissection and trypsin digestion techniques were applied for the detection of the cysts. The infected samples were fixed in glutaraldehyde and/or frozen at −20°C until use for ultrastructural and molecular studies, respectively. The ultrastructural and molecular studies were carried out contemporaneously. The 18S rRNA gene of the parasites was amplified by PCR. The PCR products were cloned into a pTZ57R/T and sequenced. In addition, the PCR products were digested separately with each of the four restriction enzymes for RFLP. Our results indicated that only microcysts were observed in muscle samples. The microcysts were white, elongated, spindled, and a few spiral-shaped, with mean size 260×75 μm which are identical with *S. cameli*. The ultrastructure of microcyst wall had many non-branched finger-like protrusions irregularly folded. There was a 600-bp specific band amplified after PCR with specific primers. The molecular data for camel *Sarcocystis* is reported for the first time in Iran and the world.

G. R. Motamedi · A. Nouri · K. Aghaeipour
Razi Vaccine and Serum Research Institute,
Tehran, Iran

A. Dalimi (✉)
Department of Parasitology, Medical Sciences Faculty,
Tarbiat Modares University,
P.O. Box 14115-111, Tehran, Iran
e-mail: dalimi_a@modares.ac.ir

Introduction

Sarcocystosis is caused by different species of *Sarcocystis*, an intracellular protozoan parasite belonging to phylum Apicomplexa. More than 150 species of *Sarcosystis* are known as parasites of domestic and wild animals. The genus is worldwide distributed. These parasites have an indirect life cycle, between a definitive and an intermediate host. Intestinal infection occurs in the definitive hosts, and tissue invasion is seen in the intermediate hosts. Some species are pathogenic organisms dangerous to humans and livestock (Butkauskas et al. 2007; Fayer 2004). *Sarcocystis* is a known parasite of considerable veterinary economic and public health importance. Intermediate hosts become infected when they ingest oocysts or sporocysts (Al-Goraishi et al. 2004). The parasite produces tissue cyst in muscles of intermediate host. The muscle cysts may be macroscopic or microscopic in size.

Sarcocystis cameli was first described in one-humped camels (*Camelus dromedarius*) in Egypt by Mason (1910), and it is the only species which have so far been reported in camels. There are only three reports on the prevalence of sarcocysts infection in camels from Tehran, Esfahan, and Khorasan Provinces in the central and northeastern area of Iran (Rahbari et al. 1981; Shekarforoush et al. 2006; Valinezhad et al. 2008). Rahbari et al. (1981) and Shekarforoush et al. (2006) examined the camel muscles by impression smear method, and they reported infection

rates of 52.6% and 52.3%, respectively. Valinezhad et al. (2008) examined the muscles by histopathological method and reported infection rates of 83.6%. They found only microcysts in camel muscles.

Ultrastructure characteristic of sarcocysts is one of the most important criteria for the specification of *Sarcocystis* species (Mehlhorn and Heydorn 1978, 1979; Mehlhorn et al. 1976; Melhorn 2008). Ultrastructural study on cysts wall has been used for identification of *Sarcocystis* species isolated from different animals by many workers (Abdel-Ghaffar et al., 2009; Al-Goraishi et al. 2004; Dalimi et al. 1999; Dubey et al. 1989a; Dubey et al. 1989b; Ghaffar et al. 1989; Mehlhorn and Heydorn 1978, 1979; Mehlhorn et al. 1976). Since studies on *Sarcocystis* taxonomy in camel are scarce and there is a lack of comprehensive information on species infecting this animal as intermediate host (Shekarforoush, et al. 2006), conducting investigation on species characterization of the parasite is so much necessary in our country. In addition, in recent years, molecular technique has been applied for *Sarcocystis* characterization isolated from different animals by some investigators (Butkauskas et al. 2007; Costa da Silva et al. 2009; Fischer and Odening 1998; Holmdahl et al. 1999; Li et al. 2002; Yang and Zuo 2000; Yang et al. 2000; Yang et al. 2001a; Yang et al. 2001b; Yang et al. 2002). As a noticeable point, there is no molecular report conducting camel *Sarcocystis* in the world.

The aim of the present study was to investigate a full description and distinguish the *Sarcocystis* species isolated from camel by studying the ultrastructure of the cyst wall by electron microscopy and combining these data with information on DNA sequence and restriction fragment length polymorphism (PCR-RFLP) characters.

Materials and methods

Sample collecting

One-humped camels (*C. dromedarius*) are raised in the semi-arid regions in Iran, mostly for transportation and meat consumption purposes. Muscle samples were taken from the fresh esophagus, diaphragm, skeletal muscles, and heart of one-humped camels slaughtered in abattoirs of Tehran and Ghazvin provinces, Iran. The specimens were tagged according to each organ. Collected samples of each organ were examined by the naked eye for macrocyst. For detection of microscopic sarcocysts in muscles, the surface of the muscles was cut serially and searched with a sterio-microscope at ×12 to ×25 magnification. Cysts were isolated from dissections using sterile needles. Fresh smear preparations by squash and digestive techniques were applied for the detection and verification of microscopic sarcocysts and existence of bradyzoites (Dubey et al. 1989a, b; Odening et al. 1996).

Squash technique

The technique was used for detection of bradyzoite, briefly; a piece of muscle sample was squashed between a cover-glass and slide and then examined unstained using a light microscope (Singh et al. 1990; Dubey et al. 2000).

Pepsin-hydrochloric acid digest

Infected organs (20 g) were incubated (20 min at 40°C) in 50 ml of acid-pepsin (2.6 g pepsin, 5 g NaCl, 7 ml HCl 1 M, 993 ml distilled water). This suspension was filtered through a 38-μm sieve, centrifuged at 2,000×*g* for 5 min, and sediment-suspended in 0.5 ml of distilled water. A drop of this solution was examined for the presence of bradyzoites with light microscope (Barham et al. 2005; Fischer and Odening 1998).

Electron microscopy

Small pieces of infected organs were washed in 0.9% NaCl, then fixed in the buffered glutaraldehyde (4%) and prepared to examine with transmission electron microscope. The samples were washed in phosphate buffer (0.2 M, pH=7.2) three times, then post fixed in osmium tetra-oxide (2% *w*/*v*, for 2 h), dehydrated in ethanol (30–100%), transferred to propylene oxide (1 h), and embedded in Epoxi Resin (EPON812), and after polymerization (72 h at 60°C), semi-thin and ultra-thin sections were cut with a Om U3 (C. Reichert, Austria) microtome. Semi-thin sections were stained with toluidine blue and examined with light microscope. Ultra-thin sections were stained with uranyl acetate and lead citrate, then examined with (Philips TEM400) transmission electron microscope (Dalimi et al. 1999).

Molecular study

Infected muscles were minced and incubated in pepsin-hydrochloric acid digest solution, and then the bradyzoites were separated from host debris by centrifugation. The sediment was washed three times with buffer saline, and cleaned zoites were pelleted in small conical tubes and held frozen at −20°C until use (Costa da Silva et al. 2009).

Total DNA was extracted from positive samples using DNA Purification Kit (DNP™KIT, CinnaGen Inc., Iran) according to the manufacturer's instruction. The DNA concentration and purity was measured with a Nano drop ND 1000 spectrophotometer. The extracted DNA was kept at −20°C until used. Polymerase chain reaction (PCR) was

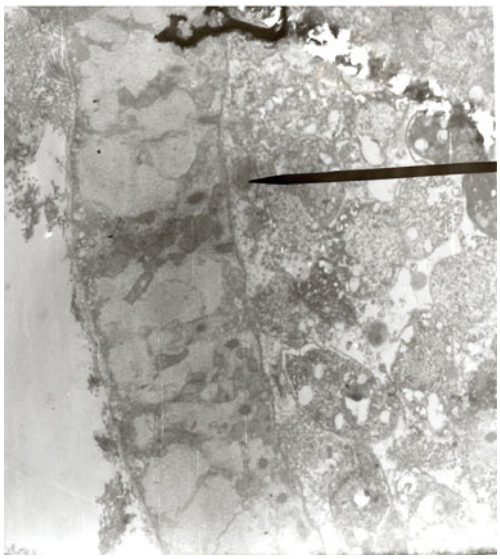

Fig. 1 Ultrastructure of primary cell wall of microcyst in *S. cameli* (magnification ×4,200)

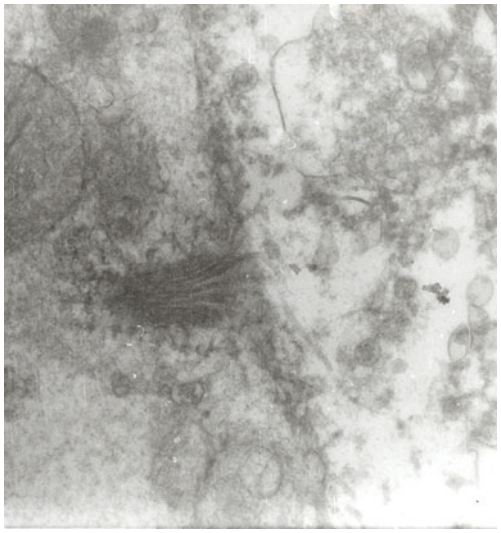

Fig. 3 Ultrastructure of fine dense homogenous granules and internal fibrillar elements of primary cell wall of microcyst in camel (magnification ×27,800)

used to amplify partial sequence of small subunit ribosomal RNA (18S rRNA) gene sequences of camel *Sarcocystis*. Forward and reverse primers were selected from domestic animals *Sarcocystis* published sequences in the gene bank. The nucleotides of primers are as:

F-5′ GCA CTT GAT GAA TTC TGG CA 3′
R-5′ CAC CAC CCA TAG AAT CAA G 3′

The PCR was performed (30 μl reactions) using 1 μl (10 pM) of each primer, 0.5 μl *Taq* polymerase, 0.5 μl dNTP, 2 μl $MgCl_2$, 10 μl DNA, 3 μl buffer 10× and 12 μl distilled water. Reactions were carried out on an Eppendorf Master Cycler Gradient thermal cycler. The PCR required a preliminary denaturation at 94°C for 5 min. The remaining PCR steps were 35 cycles at 94°C (2 min), 57°C (30 s), 72°C (2 min), with a single terminal step at 72°C (5 min). The PCR products were analyzed using agarose gel electrophoresis and purified by the PCR Purification Kit (Roche), following the manufacturer's instructions. Cloning were carried out by Plasmid Mini Extractin Kit (Bioneer, Republic of Korea) and Ins TAclone™ PCR Cloning Kit (Fermentas), respectively, following the manufacturer's instructions. The PCR products were cloned into a pTZ57R/T and sequenced. The comparison of the obtained sequences with the

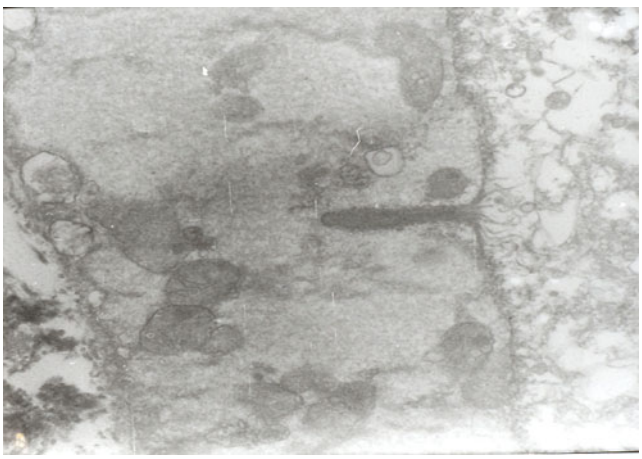

Fig. 2 Ultrastructure of primary cell wall of microcyst in *S. cameli* (magnification ×11,220)

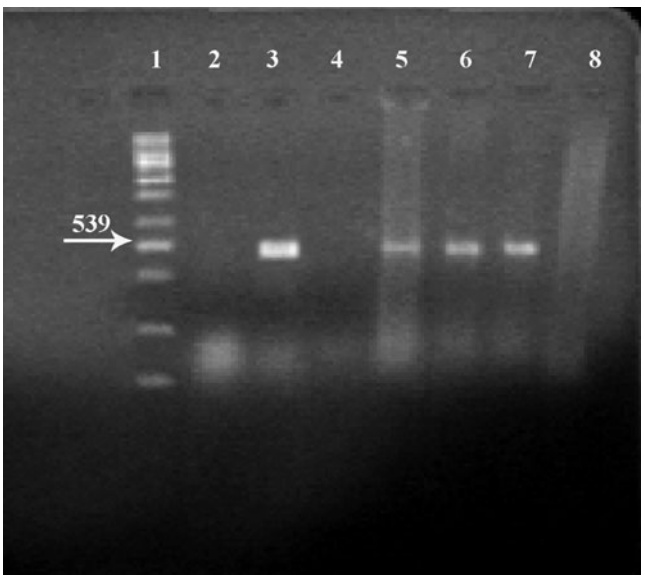

Fig. 4 PCR products of partial small subunit ribosomal RNA (18S rRNA) gene of *Sarcocystis cameli* (539 bp) as visualized on 2% agarose gels (stained with ethidium bromide) in UV light: *lane 1* DNA size marker (100 bp ladder), *lane 2* uninfected tissue (negative control), *lane 3* positive control (sheep sarcocyst), *lane 4* (negative control), *lane 5* camel sarcocyst1, *lane 6* camel sarcocyst2, *lane 7* camel sarcocyst3, *lane 8 Toxoplasma tachyzoite*

Fig. 5 Sequence of PCR product of partial small subunit ribosomal RNA (18S rRNA) gene of *Sarcocystis cameli* (539 bp)

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TATTAGGTTAGCCGTTACTTCGAGAAAATCAGAGTGTTTGAAGCAGGCTTGTTGCCTTGAATACTGCAGCATGG
AATAACAATATAGGATTTCGGTTCTATTTTGTTGGTTTCTAGAACTGGGATAATGATTAATAGGGACAGTTGGG
GGCATTTGTATTTAACTGTCAGAGGTGAAATTCTTAGATTTGTTAAAGACGAACTACTGCGAAAGCATTTGCCA
AAGATGTTTTCATTAATCAATAACGAAAGTTAGGGGCTCGAAGACGATCAGATACCGTCGTAGTCTTAACCATA
AACTATGCCGACTAGAGATAGGAAAATGTCATTTTCTGACTTCTCCTGCACCTTATGAGAAATCAAAGTCTTTG
GGTTCTGGGGGGAGTATGGTCGCAAGGGTGAAACTTAAAGGAACTGACGGAAGGGCACCACCAGGCGTGGAGCC
TGCGGCTTAATTTGACTCAACACGGGGAAACTCACCAGGTCCAGACATGGGAAGGATTGACAGATTGATAGCTC
TTTCTTGATTCTATGGGTGGT
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GenBank was performed using Blast program. After sequencing the 18S rRNA, the PCR products were digested separately with each of the four restriction enzymes (*Xba*1, *Mbo*1, *Eco*R1, *Ava*11). Each 30 μl restriction digest contained 20 μl of PCR product and 2 μl of the appropriate restriction enzyme and 3 μl associated buffer and 5 μl of distilled water. Incubation times and temperatures were 12 h and 37°C, respectively. The digests were terminated by addition of 4 μl loading mixture (50% glycerol, 0.1 M ethylene diamine tetra-acetic acid, 0.01% bromo-phenol blue, pH=8). Digestion products were electrophoressed on 2% agarose gel at 60 V (120 min). Gel was stained with ethidium bromide, and the fragments visualized with UV transilluminator. Digestion reactions containing PCR product but lacking restriction enzyme were used as negative controls. A DNA size marker was run on each gel for estimation of fragment size (Yang et al. 2002; Fischer and Odening 1998).

Results

No macroscopic sarcocysts were found in any of the samples obtained from camels (*C. dromedarius*) and only microcystic species were observed. The microcysts were elongated, spindle, and a few spiral-shaped, with a mean size 250×75 μm (305–130×105–45). In squash preparations, the bradyzoites were ovoid with mean size of 7×3 μm.

The ultastructure of the primary cyst wall revealed the presence of irregular folded non-branch finger-like villar protrusions that are characterized by the presence of internal fibrillar elements (Fig. 1). The ground substance is located directly under the primary cyst wall and extend into the protrusions and consist mainly fine dense homogenous granules and fibrillar elements which are characteristic for *S. cameli* (Figs. 2 and 3).

The PCR yielded an amplicon of approximate length of 539 bp for all samples (Fig. 4). The PCR products were cloned in a pTZ57R/T successfully and sequenced. Sequence of PCR product of partial small subunit ribosomal RNA (18S rRNA) gene of *S. cameli* is shown in Fig. 5, and restriction map of sequence of PCR product is shown in Fig. 6. The PCR amplicon was submitted in GenBank with GU074011 accession number.

Figure 7 shows the RFLP patterns for the samples after digestion with four restriction enzymes (*Xba*1, *Mbo*1, *Eco*R1, *Ava*11). *Eco*R1 does not show any restriction sites, but the other three enzymes demonstrate different size of fragments. *Xba*1 enzyme shows two fragments of 429 and 110 bp, *Mbo*1 illustrates 274 and 265 bp fragments, and *Ava*11 reveals 480 and 59 bp fragments. *Xba*1 electomorph pattern is specific for *Sarcocystis* in camel and are suitable for differentiate *S. cameli* from the rest. Thus, the PCR products of all collected samples were digested with this enzyme. Digestion with *Xba*1 yielded the same electomorph pattern for all samples, which means that the whole samples are synonym species (Fig. 8).

Discussion

Up to now, various characters such as host specificity, morphology of cyst, ultra structure of cyst wall, as well as biochemical and molecular characteristics were applied for description of *Sarcocystis* species. In fact, the cysts have

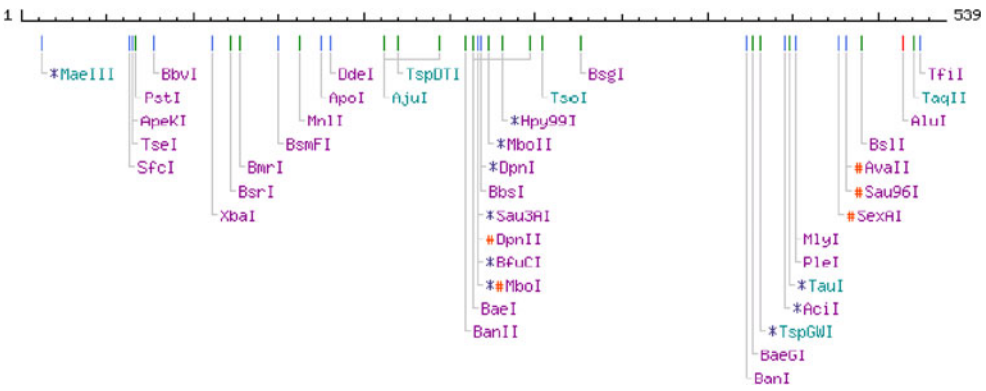

Fig. 6 Restriction map of sequence of PCR product of partial small subunit ribosomal RNA (18S rRNA) gene of *Sarcocystis cameli* (539 bp)

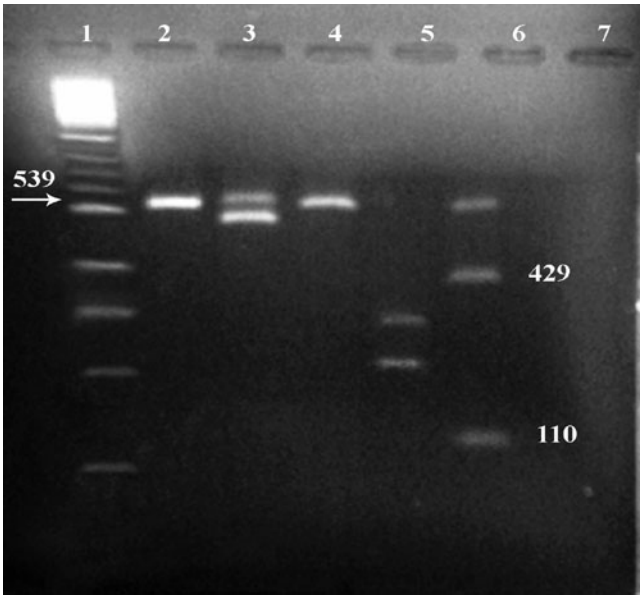

Fig. 7 RFLP pattern of PCR product of partial small subunit ribosomal RNA (18S rRNA) gene of *Sarcocystis cameli* (539 bp) with different restriction enzymes as visualized on 2% agarose gels (stained with ethidium bromide) in UV light: *lane 1* DNA size marker (100 bp ladder), *lane 2* undigested product (positive control), *lane 3* *Ava*11 enzyme digestion pattern (480 bp, 59 bp), *lane 4* *Eco*R1 enzyme digestion pattern (negative), *lane 5* *Mbo*1 enzyme digestion pattern (274 bp,265 bp), *lane 6* *Xba*1 enzyme digestion pattern (429 bp,110 bp), *lane 7* negative control

distinctive physical features that aid in species identification such as shape, overall size, and presence or absence of septa. But these features may vary with the age of sarcocyst, the host cell type, and the method of fixation.

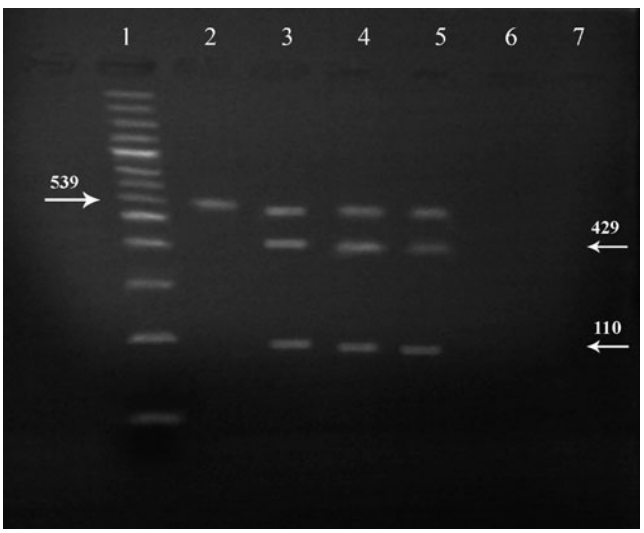

Fig. 8 *Xba*1 digest pattern of PCR product of partial small subunit ribosomal RNA (18S rRNA) gene of *Sarcocystis cameli* (539 bp) as visualized on 2% agarose gels (stained with ethidium bromide) in UV light: *lane 1* DNA size marker (100 bp ladder), *lane 2* undigested product (positive control), *lanes 3*, *4*, *5* *Xba*1 digestion pattern (429 bp, 110 bp), *lanes 6*, *7* negative control

Therefore, taxonomy based on shape and size of cyst is not reliable. Classification on the basis of the microscopic feature of cyst walls also have often failed to provide reliable characters because of unsuitable way of cyst isolation and effect of fixation substances, but the ultrastructural characters of cyst wall was found a good indication for identification of the parasite species. In addition, in recent years, molecular technique has been applied with high efficiency for species characterization of *Sarcocystis* isolated from different animals.

Although there are some reports about *Sarcocystis* species in camel, information on the developmental stages and the details of cyst wall are scarce, and only few data on camel sarcocyst ultrastructure were known. It is obvious that the *Sarcocystis* that infect camel cause microcysts which are hidden within the muscles of animal and can only be observed with armed eye. The present study showed that the examined camels have infected only with microscopic form of *Sarcocystis*. The ultrastructural observations of the cyst cell wall showed that all cysts resemble *S. cameli*. The ground substances located directly under the primary cyst wall and extend into the protrusions and consist of mainly fine dense homogenous granules and fibrillar elements are specific characters for *S. cameli* that was reported before generally. The present observation is similar to the reports of Al-Goraishi et al. (2004).

The variable regions of the 18S rRNA gene have been shown to be good genetic markers for distinguishing certain species of *Sarcocystis* (Fischer and Odening 1998; Yang and Zuo 2000). Although it is possible to study variation in this gene directly by DNA sequencing, as done by Fischer and Odening (1998); Holmdahl et al. (1999); Mugridge et al. (1999, 2000); and Yang et al. (2000, 2001a, b, 2002). On the other hand, PCR-RFLP technique have been shown to give the same result as detailed morphological studies or DNA sequence-based identifications but in a rapid and more cost-effective manner (Yang et al. 2002). Comparing the sequencing of our result with other sequences in the GenBank showed that it belonged to genus *Sarcocystis* sequences of other animals with 96–97% identity. Moreover, DNA sequencing and PCR-RFLP characters were found unique for *Sarcocystis* of camel.

In summary, the present work has demonstrated that both ultrastructural study and RFLP-based techniques may be used to distinguish camel species of *Sarcocystis* from other species. *Xba*1 is found to be an appropriate restriction enzyme to differentiate *Sarcocystis* of camel from other *Sarcocystis* in goat, sheep, and cattle. The PCR-RFLP technique is cost-effective and rapid in comparison with ultrastructural study or DNA sequencing procedures. In addition, our results indicated that only one *Sarcocystis* species (*S. cameli*) was identified in our country.

Acknowledgements This research has been supported financially by Razi Vaccine and Serum Institute and Tarbiat Modares University. The authors would like to thank the staff of Parasitology, Electron Microscopy and Biotechnology Departments of Razi Institute for their kind cooperation.

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