Title

Molecular screening approach to identify protozoan and trichostrongylid parasites infecting one-humped camels (*Camelus dromedarius*)

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Abstract

Little is known about the diversity of many parasites infecting camels, with most relying on morphological parameters. DNA extracted from different tissues (n= 90) and fecal samples (n= 101) from dromedary camels (*Camelus dromedarius*) in Egypt were screened for multiple parasites using different molecular markers. Screening of tissue samples (heart) for *Toxoplasma gondii* and *Sarcocystis* spp. was performed using *B1* and 18S rRNA gene markers, respectively. *T. gondii* was further genotyped using multiplex multilocus nested PCR-RFLP (Mn-PCR-RFLP). *Sarcocystis* was analyzed using PCR-RFLP characterization (*Xba*I and *Mbo*I restriction enzymes). A taxonomically challenging but important group of nematodes (Trichostrongylidae family) were screened using the ITS-2 ribosomal DNA (rDNA) species-specific markers. Furthermore, nested PCR was used for the detection of *Cryptosporidium* spp. (SSU rRNA gene) and positive samples were genotyped after RFLP (*Ssp*I and *Vsp*I) and sequencing. *Cryptosporidium parvum* isolates were subtyped by sequence analysis of the 60-kDa glycoprotein gene. This study revealed that many parasites infect the investigated camels, including *T. gondii* (1.1%), *Sarcocystis* spp. (64.4%), *Cryptosporidium* spp. (5.9%) and Trichostrongylidae nematodes (22.7%). The species contribution for nematodes was as follows: *Haemonchus* spp. (95.6%), *Trichostrongylus axei* (26%), *Trichostrongylus colubriformis* (65.2%) and *Cooperia oncophora* (60.8%). Mn-PCR-RFLP typing for *Toxoplasma* was only successful for three markers: 5′-SAG2 (type II), 3′-SAG2 (type II) and alt. SAG2 (type II). PCR-RFLP using *Xba*I showed possible mixed *Sarcocystis* infection. Moreover, the *Cryptosporidium* genotypes detected were *C. parvum* (IIdA19G1 and IIaA15G1R1), *Cryptosporidium* rat genotype IV and a novel genotype (camel genotype). This approach revealed the unique *Cryptosporidium* genotypes infecting the investigated camels, and the high genetic diversity of the investigated parasites.

Keywords: *Camelus dromedarius*, *Toxoplasma gondii*, *Sarcocystis*, *Cryptosporidium*, Trichostrongylidae, Egypt.

1. Introduction

The genus *Camelus* contains three species: *Camelus dromedarius* (one-hump dromedary), *Camelus bactrianus* and *Camelus bactrianus ferus* (two-hump Bactrian camel). Dromedaries are mainly found in the Middle East, and parts of Africa, south Asia and Central Australia (Kadim *et al.,* 2014; Saeed *et al.,* 2018). They are important to the economy of many countries, especially Arab countries where they are used for the production of milk, meat, wool and hides, besides their importance as draught and racing animals. The demand for camel (*C. dromedarius*) meat in Egypt and other African countries is increasing due to the great food gap.

Little is known about the genetic structure of many parasites infecting camels. The protozoan parasites *Toxoplasma gondii*, *Sarcocystis* and *Cryptosporidium* have been frequently reported in dromedary camels but molecular data on these organisms are limited. Molecular studies have been published for *T. gondii* (Abu-Zeid *et al.*, 2005 reviewed by Sharif *et al.,* 2017; Elfadaly *et al.,* 2017; Kareshk *et al.,* 2018), *Cryptosporidium* spp. (Abdel-Wahab and Abdel-Maogood, 2011; Gu *et al.,* 2016; Baroudi *et al.,* 2018; Zahedi *et al.,* 2018) and *Sarcocystis* spp. (Motamedi *et al.,* 2011). A taxonomically challenging but important pathogenic group of nematodes (the Trichostrongylidae family) have been increasingly reported in camels based on the morphological identification of adult worms, yet molecular data remain limited. Recent reports confirmed the presence of *Haemonchus longistipes* (Sultan *et al.,* 2014 and Kandil *et al.,* 2018) in infected camels by molecular methods. The objectives of the work presented herein were: 1) to determine the different genotypes of camel isolates of *T. gondii* and

Cryptosporidium, which may elucidate their role in transmission of these parasites to humans; 2) to estimate the diversity of *Sarcocystis* species isolated from slaughtered camels in Egypt; 3) to evaluate the effectiveness of a standard PCR to identify some important species of the Trichostrongylidae family infecting camels, using oligonucleotide primers employed with other ruminants.

2. Materials and methods

2.1. Animals, study area and specimens

Ninety specimens of heart tissue were obtained from slaughtered one-humped camels at Al Bassatein abattoir, Cairo, Egypt. In addition, 101 fecal samples were obtained from camels awaiting slaughter. The camels were aged 4–9 years (males and females were not distinguished), and samples were collected during the period from September 2017 to March 2018. No data were available on the source, husbandry and/or management regimens of the studied animals.

2.2. Screening heart samples for *T. gondii* and *Sarcocystis*

2.2.1. Pepsin-hydrochloric acid digestion and DNA extraction

About 30 g of each heart muscle was cut into small (1–2 cm) pieces after trimming off the connective tissue and fat. Tissues were chopped and gently ground in a blender without any fluid, then homogenized in saline (0.85% NaCl) for 30 s, mixed with acidic pepsin, and incubated in a water bath with regular shaking for 60 min at 37°C. The homogenate was filtered through two layers of gauze, centrifuged, sediment-neutralized with sodium bicarbonate, and centrifuged again (Dubey, 1998). The resulting sediment was collected in Eppendorf tubes and

stored at □20°C for DNA extraction. QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany) was used for DNA extraction following the manufacturer's protocol and DNA eluted with 100 µl AE buffer.

2.2.2. Molecular detection and characterization of *T. gondii*

PCR screening was carried out in a 20-µl total volume consisting of 2 µl of 10× PCR buffer with 15 mM $MgCl_2$, 2 µl dNTPs (2 mM), 0.4 µl (50 µM) each of the forward and reverse B1 gene primers (B22-23) (Bretagne et al., 1993), 0.2 µl of AmpliTaq Gold polymerase (5 U/µl) (Applied Biosystems, Carlsbad, CA, USA) and 200 ng of DNA template. The reaction mixture was treated at 95℃ for 5 min, followed by 40 cycles of 95℃ for 15 s, 60℃ for 15 s and 72℃ for 1 min, with a final extension step at 72℃ for 7 min. Genotyping was performed using multiplex multilocus nested PCR-RFLP (Mn-PCR-RFLP) typing for 12 different genetic markers; SAG1, SAG2 (5′-SAG2 and 3′-SAG2), alt. SAG2, SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1 and Apico as previously described by Su *et al.* (2010); Su and Dubey (2009) (Table S1). Each assay was run at least twice using separate aliquots for both detection and genotyping before samples were considered negative. Cell-cultured tachyzoites of *T. gondii* type I (RH), type II (PLK) and type III (VEG) strains were used as positive controls. To reveal the RFLP pattern of each reference strain and camel isolate, 5 µl of PCR products were mixed with 15 µl of digestion reaction containing 1× NEB buffer and restriction enzymes added according to the manufacturer's instructions (New England BioLabs, Ipswich, MA, USA) (Table S2). The digested PCR products were resolved in a 2.5% agarose gel.

2.2.3. Molecular detection and characterization of *Sarcocystis* spp.

PCR detection for *Sarcocystis* spp. was carried out using the 18S rRNA gene primer pair Sar-F1 and Sar-R1 (Motamedi *et al.,* 2011). PCR reactions were carried out in a 25-µl total volume consisting of 2.5 µl of 10× PCR buffer with 15 mM $MgCl_2$, 2.5 µl dNTPs (2 mM), 0.5 µl (50 µM) each of the forward and reverse primers, 0.2 µl of AmpliTaq Gold polymerase (5 U/µl) and 200 ng of DNA template. The reaction mixture was treated at 94℃ for 5 min, followed by 40 cycles of 94℃ for 2 min, 57℃ for 30 s and 72℃ for 2 min, with a final extension step at 72℃ for 5 min. The PCR products were digested separately with two restriction enzymes (*Xba*I, *Mbo*I). Each 20-µl restriction digest contained 10 µl of PCR product, 0.8 µl of *Mbo*I restriction enzyme (New England BioLabs), 2 µl of associated 10× NEB buffer and 7.2 µl of MilliQ water. Incubation times and temperatures were 2 h and 37°C, respectively. *Xba*I digestion was performed following the manufacturer's instructions (Takara Bio. Inc., Otsu, Japan), with a 20-µl restriction digest containing 10 µl of PCR product, 1 µl of *Xba*I restriction enzyme, 2 µl 10× M buffer, 2 µl 0.1% BSA and 5 µl of MilliQ water. Incubation times and temperatures were 12 h and 37°C, respectively.

2.3. Screening fecal samples for *Cryptosporidium* and trichostrongylid parasites

2.3.1. Sugar flotation technique for the isolation of eggs and oocysts and DNA extraction

About 5 g of each fecal sample was crushed and mixed thoroughly with distilled water to form a uniform suspension and was then immediately filtered through two layers of gauze before decanting 14 ml into a centrifuge tube, which was spun for 10 min. The supernatant was discarded and saturated sugar solution (minimum density 1.27) was added and mixed with the fecal pellet using an applicator stick. The tube was then filled within a few millimeters from the top with the sugar solution and centrifuged for 10 min. The tube was placed in a stable upright rack and filled with additional sugar solution until a convex meniscus formed, then a 22-mm coverslip was placed on top. This was allowed to sit for 1 h to allow parasitic eggs and oocysts

to float to the top of the tube and collect on the coverslip, as determined under a microscope. The coverslip was then gently removed and rinsed with 10 ml distilled water in a 50 ml tube with vigorous shaking. Then fluid was transferred to a centrifuge tube and centrifuged for 10 min (Cox and Todd, 1962). The supernatant was removed and the sediment was collected in Eppendorf tubes and stored at □20°C for DNA extraction. Using the QIAamp DNA stool Mini Kit (Qiagen), DNA was extracted following the manufacturer's protocol and DNA eluted with 100 µl AE buffer.

2.3.2. Nested PCR detection of *Cryptosporidium* and typing/subtyping analysis

An 834-bp segment of the *Cryptosporidium* small subunit (SSU) rRNA gene was amplified by nested PCR using primers previously described by Xiao *et al.* (1999[a]) and Xiao *et al.,* (2001). PCR reactions were carried out in a 25-µl total volume consisting of 2.5 µl of 10× PCR buffer with 15 mM $MgCl_2$, 2.5 µl dNTPs (2 mM), 0.5 µl (25 µM) each of the forward and reverse primers, 0.2 µl of AmpliTaq Gold polymerase (5 U/µl) and 5 µl of DNA template. The reaction mixture was treated at 94℃ for 3 min, followed by 40 cycles of 94℃ for 45 s, 55℃ for 45 s and 72℃ for 1 min, with a final extension step at 72℃ for 7 min. The secondary positive PCR products were digested separately with two restriction enzymes (*Ssp*I, *Vsp*I). Each 20-µl restriction digest contained 10 µl of PCR product, 0.8 µl of *Ssp*I restriction enzyme (New England BioLabs), 2 µl of associated 10× NEB buffer and 7.2 µl of MilliQ water. Incubation times and temperatures were 1 h and 37°C, respectively. For *Vsp*I digestion, each 20-µl restriction digest contained 10 µl of PCR product, 1 µl of restriction enzyme (Thermo Fisher Scientific, St. Louis, MO, USA), 2 µl of associated 10× Buffer O and 7 µl of MilliQ water. Incubation times and temperatures were 1 h and 37°C, respectively.

Subtyping of *Cryptosporidium parvum* isolates was achieved by amplifying a fragment of the *gp60* gene using nested PCR, with the primers described by Alves *et al*. (2003), and PCR cycling was performed as previously described (Trotz-Williams *et al.,* 2006).

2.3.3. PCR detection of trichostrongylid nematodes using species-specific primers

To assess the incidence of Trichostrongylidae species that are shared between dromedary camels and other ruminants, primers of the second internal transcribed spacer (ITS-2) region of ribosomal DNA (rDNA) were used. Five species-specific primers for *Haemonchus contortus, Teladorsagia circumcincata, Trichostrongylus axei, Trichostrongylus colubriformis* and *Cooperia oncophora*, together with a pair of generic primers (Bisset *et al.,* 2014), were used in a simplex PCR approach. The PCR reactions were carried out in a 25-µl total volume consisting of 2.5 µl of 10× PCR buffer with 15 mM $MgCl_2$, 2.5 µl dNTPs (2 mM), 0.5 µl (25 µM) each of the forward and reverse primers (Table S1), 0.2 µl of AmpliTaq Gold polymerase (5 U/µl) and 5 µl of DNA template. The reaction mixture was treated at 95℃ for 8 min, followed by 40 cycles of 94℃ for 1 min, 55℃ for 1 min and 72℃ for 2 min, with a final extension step at 72℃ for 7 min. Generic primers were firstly used to screen microscopy positive samples, then PCR reactions were performed using appropriate specific primers (forward or reverse) in combination with one of the generic primers. Moreover, a pair of primers that amplify the ITS-2 region of *Marshallagia marshalli* was used to detect this parasite, according to a protocol reported by Dallas *et al*. (2000).

2.4. Sequencing analysis

All secondary *Cryptosporidium*-positive PCR products of the SSU rRNA and *gp60* genes were cloned and sequenced, in addition to the 3′-SAG2 amplicon of *T. gondii*. To confirm

193 the specificity of the primers used for PCR detection of Trichostrongylid nematodes, between
194 two and four positive isolates were cloned and sequenced, including the ITS-2 generic products.
195 Amplicons from all selected isolates were separated by electrophoresis on 1% agarose gels and
196 bands were excised from the gel and purified using the NucleoSpin® Gel and PCR Clean-up
197 kit (Macherey-Nagel, Diiren, Germany), and their quantity and purity were evaluated using a
198 NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The
199 purified amplicons were cloned using pCR™4-TOPO® TA Cloning Kits for Sequencing
200 (Thermo Fisher Scientific, St. Louis, MO, USA). The plasmid construct was purified using the
201 QIA prep®Spin Miniprep Kit (Qiagen) and between two and five positive clones for each
202 isolate were sequenced (bidirectionally using the M13 forward and reverse primers), with the
203 Bigdye Terminator Cycle Sequencing Kit (Applied Biosystems) using a 3100 Genetic Analyzer
204 (Applied Biosystems). All sequences were aligned with each other and with reference
205 sequences obtained from the GenBank database by Basic Local Alignment Search Tool
206 (BLAST) analyses.

207 *Sarcocystis* spp. with different RFLP profiles was directly sequenced in two directions
208 using the same PCR primers. Kits used for the purification and sequencing of amplicons were
209 the same.

210 A phylogenetic tree of SSU rRNA gene sequences of *Cryptosporidium* spp. was
211 constructed using the maximum likelihood (ML) method. The nucleotide substitution models
212 with the best fit to the data set were selected using the MEGA6 software. The Tamura 3-
213 parameter model with gamma distribution (T92+G) was used. Sequences of the SSU rRNA
214 gene of *Cryptosporidium* from camels and other species were retrieved from the GenBank
215 database and were used for phylogenetic analysis. The *Eimeria tenella* sequence was retrieved
216 from the GenBank database (AF026388) and was used as outgroup.

Representative nucleotide sequences obtained in this study were deposited in the GenBank database under accession numbers MK491508 to MK491512, MK936874 to MK936889, MK948875, MK948876 and MK955943 (Table S3).

3. Results

PCR was used to detect *T. gondii* using *B1* gene primers. Only one positive sample was detected (1.1%, Table 1). Mn-PCR-RFLP typing for *T. gondii* was only successful for three markers: 5′-SAG2 (type II), 3′-SAG2 (type II) and alt. SAG2 (type II) (Fig. 1A–C). Sequencing of the 3′-SAG2 amplicon confirmed the presence of a *Hha*I restriction site, which characterized this isolate as belonging to type II. This study revealed a higher infection rate with *Sarcocystis* spp. in the investigated samples (64.4%). RFLP analysis of PCR products yielded a uniform banding pattern with two different profiles for *Xba*I; one of them was not completely digested forming three fragments and one was slightly digested (Fig. 1E). RFLP banding pattern was divided into three bands: the upper band, which was equal to the undigested product in size representing one genotype, and two additional bands representing the other genotype. *Mbo*I gave the same electromorph for all samples (Fig. 1D). From the RFLP results, all positive samples were suspected to harbor two molecularly distinct genotypes of *Sarcocystis* indicating a mixed infection. A mixed infection, as indicated by the gel banding pattern after RFLP with *Xba*I, is demonstrated by varying degrees of band strength indicating the dominance of one genotype over the other. The amplicons from six isolates representing the two RFLP profiles (*Xba*I) for camel sarcocysts were sequenced directly, the forward primer failed to generate a sequence; however, the reverse primer provided a nearly full sequence. The identifiable reverse sequence fragments (about 500 bp) were searched for *Xba*I restriction sites using NEBcutter V2.0 software (New England BioLabs) and variation was demonstrated by a one nucleotide substitution in the enzyme recognition site (TCTAGA to TCTAAA) (Figure S1A, B) generating

a restriction site for *Xba*I (TCTAGA) in one of the two genotypes. Moreover, two peaks were detected at the variable nucleotide site (G to A) (Figure S1C) in some isolates, confirming a mixed infection.

Six samples out of the 101 fecal samples (5.9%) were found to be positive for *Cryptosporidium* spp. after nested PCR screening. RFLP analysis gave clear patterns for five samples, with the same profile of *Ssp*I restriction enzyme (Fig. 1F), but showed different *Vsp*I banding patterns (Fig. 1G). *Vsp*I digestion showed that four samples were similar, but one sample was different and was suspected to be *C. parvum*. Sequencing of the partial SSU rRNA gene revealed infection with *C. parvum* (two isolates, 834 bp), *Cryptosporidium* rat genotype IV (one isolate, 830 bp) and one unknown genotype designated as camel genotype (three isolates, 827 bp) based on their identity with other isolates in the GenBank database. Two haplotypes of *C. parvum* were detected with a single nucleotide polymorphism at position 435 (T to C). Two haplotypes were detected of the camel genotype with two nucleotide polymorphisms at positions 307 (G to A) and 494 (C to T). Sequence analysis of the *gp60* gene revealed two *C. parvum* subtypes, IIdA19G1 (860 bp) and IIaA15G1R1 (866 bp). Phylogenetic analysis showed that *Cryptosporidium* species formed two clusters that were statistically different. One group contained *C. parvum* and the new genotypes detected in this study (intestinal group), while the other group containing *C. muris* and *C. andersoni* (gastric group). *Cryptosporidium* spp. designated as camel genotype formed a separate branch on phylogenetic analysis (novel genotype) (Fig. 2).

In our study, trichostrongyle eggs were detected microscopically in 35 floats out of 101 examined samples (34.6%), in addition to *Trichuris* eggs. PCR screening using ITS2 generic primers detected only 23 positive samples (22.7%). The species contribution of nematodes was as follows: *Haemonchus* sp. (95.6%), *Trichostrongylus colubriformis* (65.2%), *Cooperia oncophora* (60.8%) and *Trichostrongylus axei* (26%). Mixed infections with these detected

nematodes were highly prevalent (73.9%). The ITS-2 sequences revealed a high frequency of minor differences represented by substitutions of one or more residues, while no insertions or deletions were detected (Table S3, Fig. S2). A total of 15 haplotypes were detected comprising *Trichostrongylus axei* (four haplotypes, two of 377 bp and two of 186 bp), *H. longistipes* (three haplotypes, 379 bp), *T. colubriformis* (five haplotypes, 232 bp) and *C. oncophora* (three haplotypes, 173 bp). In terms of *Haemonchus* infection, samples that tested positive using the generic primer pair were cloned and sequenced and identified as *H. longistipes*, sharing 99%–100% homology with species-specific sequences in the GenBank database. Maximum genetic diversity was detected in the *T. colubriformis* ITS-2 partial sequences (232 bp) with up to five nucleotide substitutions.

4. Discussion

It is important to investigate the genotypes of *T. gondii* in different food animals to elucidate potential links with disease manifestations in human toxoplasmosis (Su et al., 2006). Only one positive sample was detected among screened heart samples. Tissue samples were limited in both volume and the amount of parasite DNA present, particularly not from clinical cases or mouse bioassays. To alleviate this problem, a highly-sensitive method (Mn-PCR-RFLP) employing 12 genetic markers (Su and Dubey, 2009; Su *et al.,* 2010) was used for genotyping the single positive isolate. Mn-PCR-RFLP typing for *T. gondii* was only successful for three markers: 5′-SAG2 (type II), 3′-SAG2 (type II) and alt. SAG2 (type II). One possible explanation for this is that the *T. gondii* tissue burden is very low in naturally-infected camels. Previous studies on *T. gondii* genotyping in camels reported type I, II (Abu-Zeid *et al.,* 2005), type II, III (Elfadaly *et al.,* 2017) and type I, II, III (Kareshk *et al.,* 2018), but lacked specific criteria for the genotyping method and depending on single locus. Further studies should be carried out to genotype this parasite in dromedary camels because of the limitations of markers

used in our study and previous studies. Moreover, isolation of the parasite is recommended due to the possibility of lower parasitic burden.

*Xba*I RFLP analysis of *Sarcocystis* PCR products indicated that positive samples were suspected to harbor two molecularly distinct genotypes of *Sarcocystis* in mixed infection. Structural studies revealed the lack of comprehensive information on the taxonomy of *Sarcocystis* spp. in camels. Two structurally distinct *Sarcocystis* species infect this animal, namely *Sarcocystis cameli* and *Sarcocystis ippeni*, based on transmission electron microscopy (Dubey *et al.,* 2015). Furthermore, Motamedi *et al.* (2011) reported the first molecular characterization of *S. cameli* from dromedary camels in Iran and represented *Xba*I as an appropriate restriction enzyme to differentiate *S. cameli* but the *Xba*I electromorph pattern in their study may be of mixed infection (three fragments) instead of a single *S. cameli* infection (two fragments). These discrepancies reveal that molecular differentiation between *Sarcocystis* spp. infecting one-humped camels is still problematic and single cyst isolation is required coupled with ultrastructural studies in an integrated approach.

Cryptosporidiosis is a zoonotic protozoan disease of a wide range of vertebrates worldwide that causes a threat to human and animal health (Xiao *et al.,* 1999[a] and Xiao *et al.,* 2001; Xiao *et al.,* 2002; Fayer 2010; Ryan *et al.,* 2016). Molecular and phylogenetic analyses of the SSU rRNA gene have been widely used for genotyping *Cryptosporidium* species (Xiao L., 2010; Checkley et al., 2015; Khan et al., 2018). Little is known about *Cryptosporidium* species infecting camels (Table 2). Sequencing of partial SSU rRNA gene revealed infection with *C. parvum* (two isolates, 834 bp), *Cryptosporidium* rat genotype IV (one isolate, 830 bp) and one unknown genotype designed as camel genotype (three isolates, 827 bp). One haplotype of *C. parvum* showed 100% identity with a *C. parvum* cattle genotype and *C. parvum* from a dromedary camel in Algeria (accession no. LC270282). Another haplotype shared 100% identity with *C. parvum* from deer in China (accession no. KX259140). *Cryptosporidium* rat

317 genotype IV shared 100% similarity with the genotype of brown rats (*R. norvegicus*) in China
318 (MG917670) and 99.73% similarity with isolate MG917671 (Zhao *et al.,* 2018), as well as
319 99.73% similarity with sequences from *R. norvegicus* isolated in Sweden (accession no.
320 JN172970) and 99.9% identity with different isolates from environmental samples of unknown
321 animals (accession no. KY483983, JQ413365, AY737581 and AY737578). Two haplotypes
322 were detected for the novel genotype (camel genotype), which shared 97%–98.6% identity with
323 other *Cryptosporidium* species. *C. parvum* was further subtyped into IIdA19G1 (860 bp), which
324 shared 100% identity (accession no. KM199738-KT235713 and others), and IIaA15G1R1 (866
325 bp), which shared 99.88% identity, with a buffalo isolate from Egypt (accession no.
326 AB712392). The two identified *C. parvum* subtypes found in dromedary camels in this study
327 have also been found in humans. For example, IIaA15G1R1 subtype was detected in humans
328 from Egypt (Helmy *et al.,* 2013) and other Arabian countries, such as Kuwait (Sulaiman *et al.,*
329 2005) and Jordan (Hijjawi *et al.,* 2010), while being most common in human cases in Scotland
330 (Deshpande *et al.,* 2015). IIdA19G1 subtype was isolated from humans in the Netherlands
331 (Roelfsema *et al.,* 2016), Sweden (Insulander *et al.,* 2013) and Portugal (Alves *et al.* 2006). The
332 demonstration of zoonotic *C. parvum* in our study and previous studies in dromedary camels
333 (Baroudi *et al.,* 2018; Zahedi *et al.,* 2018) suggests that dromedaries can be a potential source
334 of human cryptosporidiosis. The phylogenetic relationship among *Cryptosporidium* genotypes
335 found in camels suggested the diversity of this parasite and the susceptibility of camels not only
336 to camel-specific parasites but also to a wide range of parasites from other animals.

337 The family Trichostrongylidae includes the most pathogenic and economically
338 important nematode parasites of ruminants. Knowing which of these species are infecting
339 animals is extremely important due to differences in their pathogenicity and susceptibility to
340 anthelmintic drugs (Schnieder *et al.,* 1999; Humbert *et al.,* 2001; Jurasek *et al.,* 2010). The
341 similarities in size and shape of the eggs of different species of gastrointestinal nematodes make

342 their differentiation extremely difficult. Furthermore, the identification of third stage infective
343 larvae in cultured ruminant feces is challenging (Bowman 2014) and requires an experienced
344 worker to distinguish between the different genera (Taylor, 2010; Roeber *et al.,* 2013; Roeber
345 and Kahn, 2014). The development of molecular assays has been shown to be useful in the
346 specific identification of nematodes (Christensen *et al.,* 1994; Callaghan and Beh, 1994). ITS-
347 2 has been proven to be a suitable region for species differentiation of the family
348 Trichostrongylidae (Gasser *et al.,* 1993; Newton *et al.,* 1998; Chilton *et al.,* 1998; Heise *et al.,*
349 1999; Schnieder *et al.,* 1999; Dallas *et al.,* 2000; Zarlenga *et al.,* 2001; von Samson-
350 Himmelstjerna *et al.,* 2002; Wimmer *et al.,* 2004; Harmon *et al.,* 2007; Bisset *et al.,* 2014).

351 Morphological studies have shown that dromedary camels are parasitized by various
352 trichostrongyles which are shared between dromedary camels and other ruminants, even the
353 formerly camel-specific *H. longistipes* has been recorded in cattle and sheep (Kumsa *et al.,*
354 2008; Hussain *et al.,* 2014). There have been no attempts to use PCR for the antemortem
355 diagnosis of trichostrongyles from naturally-infected dromedary camels. In this study, we
356 evaluated the effectiveness of a standard PCR in identifying some important species of this
357 family. Oligonucleotide primers used in this study were tested by BLAST and ClustalW
358 alignment with ITS-2 (rDNA) sequences retrieved from the GenBank database. The specificity
359 of the selected primers was confirmed, with the exception of the *H. contortus* forward (Fd3)
360 primer. Single internal primer–template mismatches were detected between Fd3 and *H.*
361 *longistipes* sequences, but these types of mismatches had no significant effect on the PCR
362 product yield (Kwok *et al.,* 1990). In our study, PCR screening of microscopically-positive
363 trichostrongyles samples using ITS2 generic primers detected 23/35 positive samples, which
364 may be due to the lower numbers of recovered eggs or the possibility that samples were infected
365 with other trichostrongyles that were not detectable using these primers. In agreement with
366 some previous studies, *Haemonchus* spp. was the most prevalent trichostrongyles in dromedary

camels (Haroun *et al.,* 1996; Anwar and Hayat, 1999; Bekele, 2002). Generic primer amplification of *Haemonchus*-positive samples and cloning/sequencing revealed *H. longistipes* alone, confirming the lower specificity of the HacoFd3 primer. ClustalW alignment of *H. longistipes* haplotype sequences obtained in this study (accession no. MK936878– MK936880, 379 bp) along with products amplified using the primer pair HacoFd3 - ITS-2 GR (MK936881, 90 bp), and the three primers ITS-2 GF, ITS-2 GR and HacoFd3, revealed single internal primer–template mismatches (Figure S2). Although *H. longistipes* is the most common trichostrongylid in camels, morphological studies also revealed *H. contortus* infection in dromedary camels (Anwar and Hayat, 1999; Anvari-Tafti et al., 2013). Intraspecies diversity was slightly higher in the detected trichostrongyles, and was highest for *T. colubriformis* (five substitutions, 2%), which may be attributed to the mixing of camels from different herds before slaughtering and the great genetic variability of these nematodes in comparison with other parasites (Braisher *et al.,* 2004). Our results were promising in terms of the detection and characterization of camel nematode parasites which are shared between all ruminants. This approach will elucidate the role of camels in the transmission of these nematodes by antemortem diagnosis, which in turn may help in the development of control strategies against nematodes, especially anthelmintic-resistant species. An important next step will be to couple molecular and morphological data in the investigation of trichostrongylid nematodes infecting one humped dromedary camels. This may allow a wider spectrum of species to be characterized, especially those that are more abundant in camels such as *H. longistipes* and *Camelostrongylus mentulatus*.

5. Conclusion

These data showed that camels are promising animals in parasitology research and further efforts are needed to characterize parasites infecting camels by molecular methods. Our

approach revealed unique *Cryptosporidium* genotypes infecting camels and high genetic diversity of the investigated parasites. Moreover, this study revealed the role of camels in the transmission of these parasites to other animals and humans, as some parasites (e.g., *T. gondii*, *C. parvum*, *T. axei* and *T. colubriformis*) were found to be of zoonotic importance.

Conflict of interest

No financial or personal conflicts have been declared by the authors that could negatively influence their contributions to this study.

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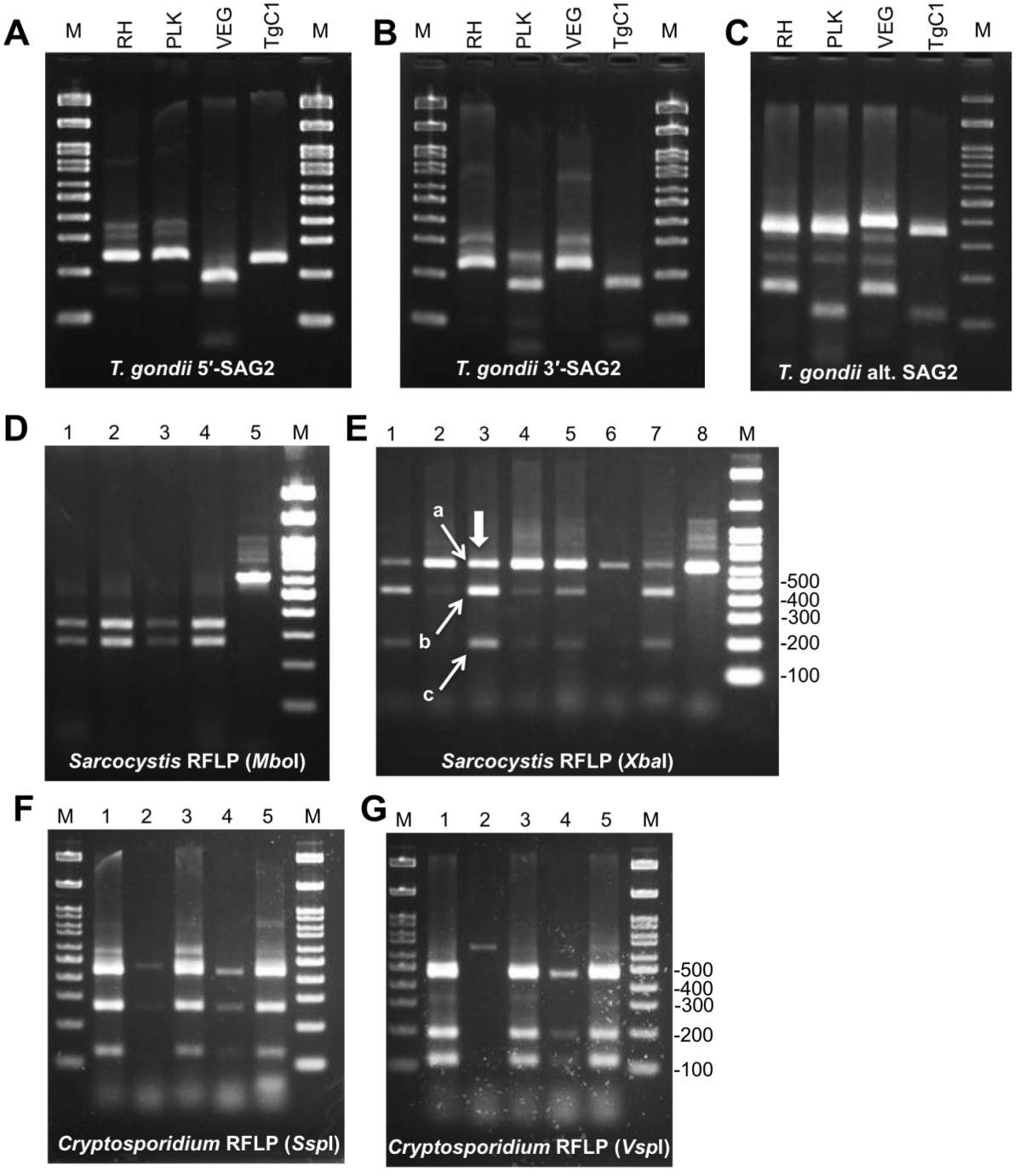
613

614 **Figure legends**

615 **Figure 1** RFLP profiles for *T. gondii* (A, B, C), *Sarcocystis* (D, E) and *Cryptosporidium* (F, G).
616 Three markers, 5′-SAG2 (A), 3′-SAG2 (B) and alt. SAG2 (C), were used for genotyping of *T.*
617 *gondii* camel isolates. RH (type I), PLK (type II), VEG (type III) and TgC1 (camel isolate) were
618 analyzed for the three markers. (D) *Mbo*I digestion of *Sarcocystis* isolates (lanes 1–4) with a
619 non-digested isolate control (lane 5). (E) *Xba*I digestion of *Sarcocystis* isolates (lanes 1–7) with
620 a non-digested isolate control (lane 8). The thick arrow in electrograph E represents the typical
621 *Xba*I pattern consisting of three bands (thin arrows): the upper band (a), which was equal to the
622 undigested product in size, and two additional bands (b, c) representing the digested product.
623 Five *Cryptosporidium* isolates were used to determine the *Ssp*I (F) and *Vsp*I (G) banding
624 patterns of the camel genotype (1, 3, 4), *C. parvum* (2) and *C.* rat genotype IV (5). M: DNA
625 marker (bp).

626

627 **Figure 2** Maximum likelihood tree based on the SSU rRNA gene sequences of
628 *Cryptosporidium*, including *Cryptosporidium* from camels. Sequences from this study are
629 labeled. The Tamura 3-parameter method, modeled using gamma distribution was employed.
630 Numbers at the nodes represent the bootstrap values with more than 50% bootstrap support
631 from 1000 pseudoreplicates.


A
M
RH
PLK
VEG
TgC1
M
T. gondii 5′-SAG2
B
M
RH
PLK
VEG
TgC1
M
T. gondii 3′-SAG2
C
RH
PLK
VEG
TgC1
M
T. gondii alt. SAG2
D
1 2 3 4 5 M
Sarcocystis RFLP (MboI)
E
1 2 3 4 5 6 7 8 M
a
b
c
-500
-400
-300
-200
-100
Sarcocystis RFLP (XbaI)
F
M 1 2 3 4 5 M
Cryptosporidium RFLP (SspI)
G
M 1 2 3 4 5 M
-500
-400
-300
-200
-100
Cryptosporidium RFLP (VspI)

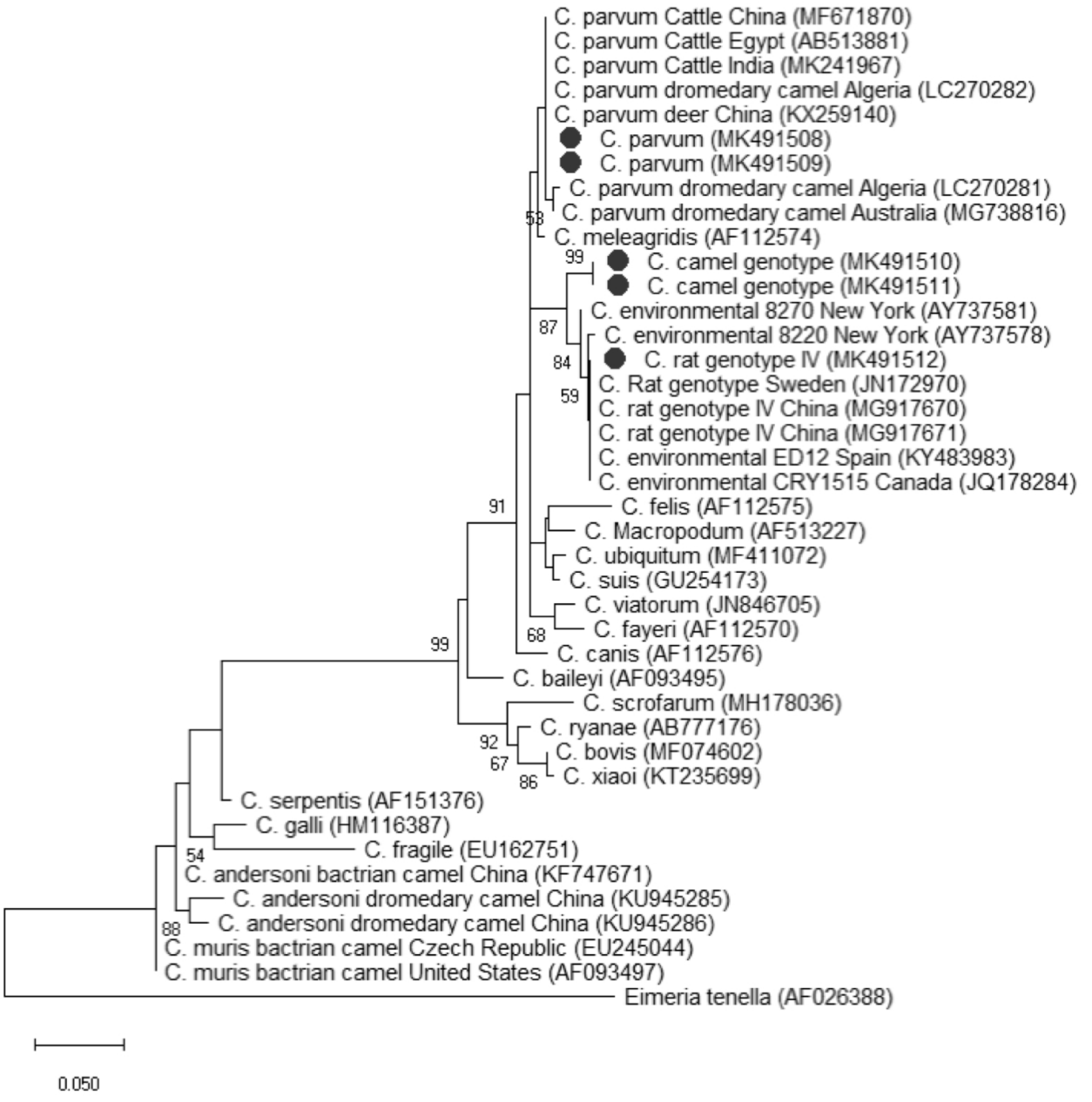

C. parvum Cattle China (MF671870)
C. parvum Cattle Egypt (AB513881)
C. parvum Cattle India (MK241967)
C. parvum dromedary camel Algeria (LC270282)
C. parvum deer China (KX259140)
C. parvum (MK491508)
C. parvum (MK491509)
C. parvum dromedary camel Algeria (LC270281)
C. parvum dromedary camel Australia (MG738816)
C. meleagridis (AF112574)
C. camel genotype (MK491510)
C. camel genotype (MK491511)
C. environmental 8270 New York (AY737581)
C. environmental 8220 New York (AY737578)
C. rat genotype IV (MK491512)
C. Rat genotype Sweden (JN172970)
C. rat genotype IV China (MG917670)
C. rat genotype IV China (MG917671)
C. environmental ED12 Spain (KY483983)
C. environmental CRY1515 Canada (JQ178284)
C. felis (AF112575)
C. Macropodum (AF513227)
C. ubiquitum (MF411072)
C. suis (GU254173)
C. viatorum (JN846705)
C. fayeri (AF112570)
C. canis (AF112576)
C. baileyi (AF093495)
C. scrofarum (MH178036)
C. ryanae (AB777176)
C. bovis (MF074602)
C. xiaoi (KT235699)
C. serpentis (AF151376)
C. galli (HM116387)
C. fragile (EU162751)
C. andersoni bactrian camel China (KF747671)
C. andersoni dromedary camel China (KU945285)
C. andersoni dromedary camel China (KU945286)
C. muris bactrian camel Czech Republic (EU245044)
C. muris bactrian camel United States (AF093497)
Eimeria tenella (AF026388)
99
58
87
84
59
91
99
68
92
67
86
54
88
0.050

Table 1. PCR detection rate of different parasites in this study

Incidence of tissue parasites

Name	**examined**	**positive**	**%**
T. gondii	90	1	1.1
Sarcocystis spp.	90	58	64.4

Incidence of GIT parasites

Name	**examined**	**positive**	**%**
Cryptosporidium spp.	101	6	5.9
Trichostongyles	101	23	22.7
Haemonchus spp.	*23	22	95.6
Teladorsagia circumcincata	*23	0	0.0
Trichostrongylus axei	*23	6	26.0
Trichostrongylus colubriformis	*23	15	65.2
Cooperia oncophora	*23	14	60.8
Marshallagia marshalli	*23	0	0.0
Mixed trichostongyles	*23	17	73.9

* indicates the contribution of each nematode species to the total number of positives not the total number of examined samples.

Table 2. Summary of genotyping reports for *T. gondii*, *Cryptosporidium* and *Sarcocystis* parasites in old world camelids

Animal species	***Cryptosporidium* species**	**Location**	**Diagnostic method**	**Reference**
Dromedary camel	*C. muris*	Egypt	PCR	Abdel-Wahab and Abdel-Maogood 2011
Dromedary camel	*C. andersoni*	China	PCR-RFLP, and sequence analysis of 18S rRNA gene	Gu *et al.,* 2016
Dromedary camel	*C. parvum* (*C. hominis* If subtype-like)	Algeria	PCR-RFLP, and sequence analysis of 18S rRNA and *gp60* gene	Baroudi *et al.,* 2018
Dromedary camel	*C. parvum* (IIaA17G2R1)	Australia	sequence analysis of multiple loci; 18S rRNA, actin and *gp60* loci	Zahedi *et al.,* 2018
Dromedary camel	*C. parvum* (IIdA19G1- IIaA15G1R1), C. rat genotype IV and new genotype	Egypt	PCR-RFLP, and sequence analysis of the partial 18S rRNA and *gp60* genes	This study
Bactrian camel	*C. muris*	United States	PCR-RFLP, and sequence analysis of 18S rRNA gene	Xiao *et al.,* 1999[b]
Bactrian camel	*C. muris*	Czech Republic	sequence analysis of two loci; 18S rDNA, and HSP-70	Morgan *et al.,* 2000
Bactrian camel	*C. muris*	Czech Republic	sequence analysis of the partial 18S rRNA gene	Kvac *et al.,* 2008
Bactrian camel	*C. andersoni*	China	sequence analysis of multiple loci; 18S rRNA, HSP70 , actin and COWP	Wang *et al.,* 2008
Bactrian camel	*C. andersoni*	China	PCR-RFLP and DNA sequence analysis of the partial 18S rRNA, COWP, and A135 genes	Liu *et al.,* 2014
Animal species	**Other species**	**Location**	**Diagnostic method**	**Reference**
Dromedary camel	*T. gondii* (type I, II)	UAE	N-PCR-RFLP SAG2	Abu-Zeid *et al.,* 2005
Dromedary camel	*T. gondii* (type II, III)	Egypt	N-PCR-RFLP SAG2 (5′-SAG2, 3′-SAG2)	Elfadaly *et al.,* 2017
Camels	*T. gondii* (type I, II, III)	Iran	N-PCR-RFLP GRA6	Kareshk *et al.,* 2018
Dromedary camel	*T. gondii* (type II)	Egypt	N-PCR-RFLP SAG2 (5′-SAG2, 3′-SAG2) and alt. SAG2	This study
Dromedary camel	*Sarcocystis cameli*	Iran	18S rRNA PCR; RFLP *(Xba*1) and sequencing	Motamedi *et al.,* 2011
Dromedary camel	*Sarcocystis* spp.	Egypt	18S rRNA PCR; RFLP (*Xba*1)	This study

Supplementary material

Molecular screening approach to identify protozoan and trichostrongylid parasites infecting one-humped camels (*Camelus dromedarius*)

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Table S1. Primer sequences used in this study

Markers	PCR primers (external primers)	Nested PCR primers (internal primers)	Amplicon size (bp)	Target	References
B1	F: AACGGGCGAGTAGCACCTGAGGAGA R: TGGGTCTACGTCGATGGCATGACAAC	-	90	*T. gondii*	Bretagne et al., 1993
SAG1	F: GTTCTAACCACGCACCCTGAG R: AAGAGTGGGAGGCTCTGTGA	F: CAATGTGCACCTGTAGGAAGC R: GTGGTTCTCCGTCGGTGTGAG	390	*T. gondii*	Grigg *et al.,* 2001; Su and Dubey, 2009; Su et al. 2010
5′-SAG2	Covered by the external primers of alt. SAG2	F: GAAATGTTTCAGGTTGCTGC R: GCAAGAGCGAACTTGAACAC	242	*T. gondii*	Howe *et al.,* 1997; Su *et al.,* 2006; Su and Dubey, 2009; Su *et al.,* 2010
3′-SAG2	F: TCTGTTCTCCGAAGTGACTCC R: TCAAAGCGTGCATTATCGC	F: ATTCTCATGCCTCCGCTTC R: AACGTTTCACGAAGGCACAC	222	*T. gondii*	Howe *et al.,* 1997; Su and Dubey, 2009; Su *et al.,* 2010
alt. SAG2	F: GGAACGCGAACAATGAGTTT R: GCACTGTTGTCCAGGGTTTT	F: ACCCATCTGCGAAGAAAACG R: ATTTCGACCAGCGGGAGCAC	546	*T. gondii*	Khan *et al.,* 2005; Su *et al.,* 2006; Su and Dubey, 2009; Su *et al.,* 2010
SAG3	F: CAACTCTCACCATTCCACCC R: GCGCGTTGTTAGACAAGACA	F: TCTTGTCGGGTGTTCACTCA R: CACAAGGAGACCGAGAAGGA	225	*T. gondii*	Grigg *et al.*, 2001; Su and Dubey, 2009; Su *et al.,* 2010
BTUB	F: TCCAAAATGAGAGAAATCGT R: AAATTGAAATGACGGAAGAA	F: GAGGTCATCTCGGACGAACA R: TTGTAGGAACACCCGGACGC	411	*T. gondii*	Khan *et al.,* 2005; Su *et al.,* 2006; Su and Dubey, 2009; Su *et al.,* 2010
GRA6	F: ATTTGTGTTTCCGAGCAGGT R: GCACCTTCGCTTGTGGTT	F: TTTCCGAGCAGGTGACCT R: TCGCCGAAGAGTTGACATAG	344	*T. gondii*	Khan *et al.,* 2005; Su *et al.,* 2006; Su and Dubey, 2009; Su *et al.,* 2010
C22-8	F: TGATGCATCCATGCGTTTAT R: CCTCCACTTCTTCGGTCTCA	F: TCTCTCTACGTGGACGCC R:AGGTGCTTGGATATTCGC	521	*T. gondii*	Khan *et al.,* 2005; Su *et al.,* 2006; Su and Dubey, 2009; Su *et al.,* 2010
C29-2	F: ACCCACTGAGCGAAAAGAAA R: AGGGTCTCTTGCGCATACAT	F: AGTTCTGCAGAGTGTCGC R:TGTCTAGGAAAGAGGCGC	446	*T. gondii*	Khan *et al.,* 2005; Su *et al.,* 2006; Su and Dubey, 2009; Su *et al.,* 2010
L358	F: TCTCTCGACTTCGCCTCTTC R: GCAATTTCCTCGAAGACAGG	F: AGGAGGCGTAGCGCAAGT R: CCCTCTGGCTGCAGTGCT	418	*T. gondii*	Khan *et al.,* 2005; Su *et al.,* 2006; Su and Dubey, 2009; Su *et al.,* 2010
PK1	F: GAAAGCTGTCCACCCTGAAA R: AGAAAGCTCCGTGCAGTGAT	F: CGCAAAGGGAGACAATCAGT R: TCATCGCTGAATCTCATTGC	903	*T. gondii*	Khan *et al.,* 2005; Su *et al.,* 2006; Su and Dubey, 2009; Su *et al.,* 2010
Apico	F: TGGTTTTAACCCTAGATTGTGG R: AAACGGAATTAATGAGATTTGAA	F: GCAAATTCTTGAATTCTCAGTT R: GGGATTCGAACCCTTGATA	640	*T. gondii*	Su et al. 2006; Su and Dubey, 2009; Su *et al.,* 2010
SSU rRNA gene	F: TTCTAGAGCTAATACATGCG R: CCCATTTCCTTCGAAACAGGA	F: GGAAGGGTTGTATTTATTAGATAAAG R: AAGGAGTAAGGAACAACCTCCA	834	*Cryptosporidium* spp.	Xiao *et al.,* 1999[a]; Xiao *et al.,* 2001
gp60 gene	F: ATAGTCTCCGCTGTATTC R: GGAAGGAACGATGTATCT	F: TCCGCTGTATTCTCAGCC R: GCAGAGGAACCAGCATC	~ 850	*Cryptosporidium* spp.	Alves *et al.* (2003)
ITS-2 rDNA	GF: CACGAATTGCAGACGCTTAG GR: GCTAAATGATATGCTTAAGTTCAGC	-	370-398	Trichostrongyle nematodes	Bissetet *et al.,* 2014
	HacoFd3: CATGTATGGCGACGATGTTCTT	-	90	*Haemonchus* spp.	Bisset *et al.,* 2014
	TrcoRv1: ACATCATACAGGAACATTAATGTCA	-	232	*Trichostrongylus colubriformis*	Bisset *et al.,* 2014
	TraxFd2: GATGTTAATGTTGAACGACATTAATATC	-	186	*Trichostrongylus axei*	Bisset *et al.,* 2014
	TeciFd3: AAACTACTACAGTGTGGCTAACATA	-	295-297	*Teladorsagia circumcincata*	Bisset *et al.,* 2014
	CoonRv1: CTATAACGGGATTTGTCAAAACAGA	-	173	*Cooperia oncophora*	Bisset *et al.,* 2014
	Mm-ITS2-F: TCCTGAATGATATGAATGTATTACC Mm-ITS2-R: CAATACAAATGATATATTGAACATACAG	-	90	*Marshallagia marshalli*	Dallas *et al.,* 2000
18S rRNA gene	F: GCACTTGATGAATTCTGGCA R: CACCACCCATAGAATCAAG	-	~ 600	*Sarcocystis* spp.	Motamedi *et al.*, 2011

Table S2. Restriction enzymes used in this study

Markers	**Restriction enzymes and manufacturer**	**Incubation temp. and time**	**Target parasite**
5′-SAG2	*Mbo*I, (New England BioLab, Ipswich, MA, USA)	37℃ for 1hr	*T. gondii*
3′-SAG2	*Hha*I, (New England BioLab, Ipswich, MA, USA)	37℃ for 1hr	*T. gondii*
alt. SAG2	*Hinf*I+*Taq*I(double digest), (New England BioLab, Ipswich, MA, USA)	37℃ and 65℃ for 30 min and 30 min.	*T. gondii*
SSU rRNA gene	*Ssp*I, (New England BioLab, Ipswich, MA, USA) *Vsp*I, (Thermo Fisher Scientific, St. Louis, MO, U.S.A.)	37℃ for 1hr 37℃ for 1hr	*Cryptosporidium spp.*
18S rRNA gene	*Xba*I, (Takara bio Inc, Japan). *Mbo*I, (New England BioLab, Ipswich, MA, USA)	37℃ for 12hr 37℃ for 1hr	*Sarcocystis* spp.

Table S3. BLAST identification and accession numbers assigned to the isolates.

Parasite	Target gene	No. of haplotypes	Accession number	Sequence size (bp)	%identity	Genebank Accession numbers
C. parvum	SSU rRNA	2	MK491508	834	100	MK241967- MF671870 and others
			MK491509	834	100[1]- 99.88[2]	KX259140[1]-MK241967 and others[2]
C. parvum	*gp60*	IIdA19G1 (1)	MK948875	860	100	KM199738-KT235713 and others
		IIaA15G1R1 (1)	MK948876	866	99.88[1]-99.65[2]	AB712392[1]-KF128738[2]
C. camel genotype	SSU rRNA	2	MK491510	827	98.48	JQ178284
			MK491511	827	98.60	AY737581- AY737582
C. rat genotype IV	SSU rRNA	1	MK491512	830	100[1]-99.75[2]- 99.73[3]	MG917670[1]-JN172970[2]- MG917671[3]
Trichostrongylus axei	ITS-2 rDNA	4	MK936874	377	100	KC998724- KC337066 and others
			MK936875	377	100[1]-99.73[2]	JQ889794[1]-KC998724[2]
			MK936876	186	98.39	KY355033- KF880746 and others
			MK936877	186	99.46	KY355033- KF880746 and others
Haemonchus longistipes	ITS-2 rDNA	3	MK936878	379	98.94	AB682685- AB682684- AB682683
			MK936879		100[1]-99.73[2]	KU891905 and others[1]-AB682685[2]
			MK936880		99.66[1]-99.47[2]	KU891905[1]-AB682685[2]
Trichostrongylus colubriformis	ITS-2 rDNA	5	MK936882	232	100[1]-98.71[2]	JF276021[1]-KR002111 and others [2]
			MK936883		98.71	KR002111 and others
			MK936884		99.14	KR002111 and others
			MK936885		99.31[1]- 98.71[2]	JF276021[1]- KR002111 and others[2]
			MK936886		100[1]- 99.14[2]	JF276021[1]- KR002111 and others [2]
Cooperia oncophora	ITS-2 rDNA	3	MK936887	173	99.42[1]-98.84[2]	KC998744[1]-MH267774 and others[2]
			MK936888		100	MH267774- KC998743 and others
			MK936889		99.42	MH267774- KC998743 and others
T. gondii	3'-SAG2	1	MK955943	222	100	MH606150

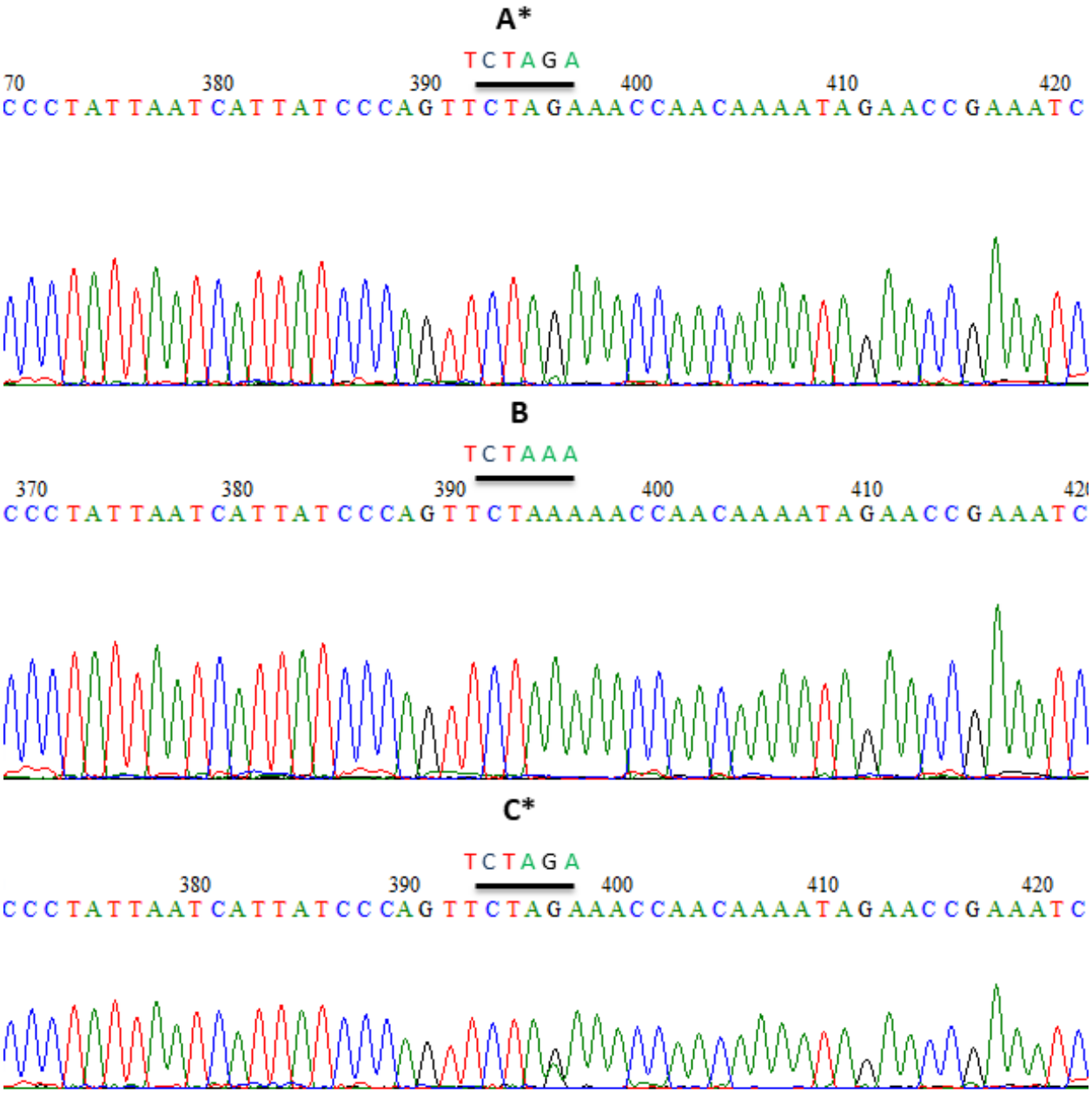

Figure S1 Chromatogram surrounding the *Xba*I restriction site within the 18S rRNA sequence of *Sarcocystis* DNA extracted from the studied camels. Horizontal bars are located over the nucleotides of interest that exhibit polymorphisms used to distinguish haplotypes. Asterisks indicate *Xba*I restriction sites.

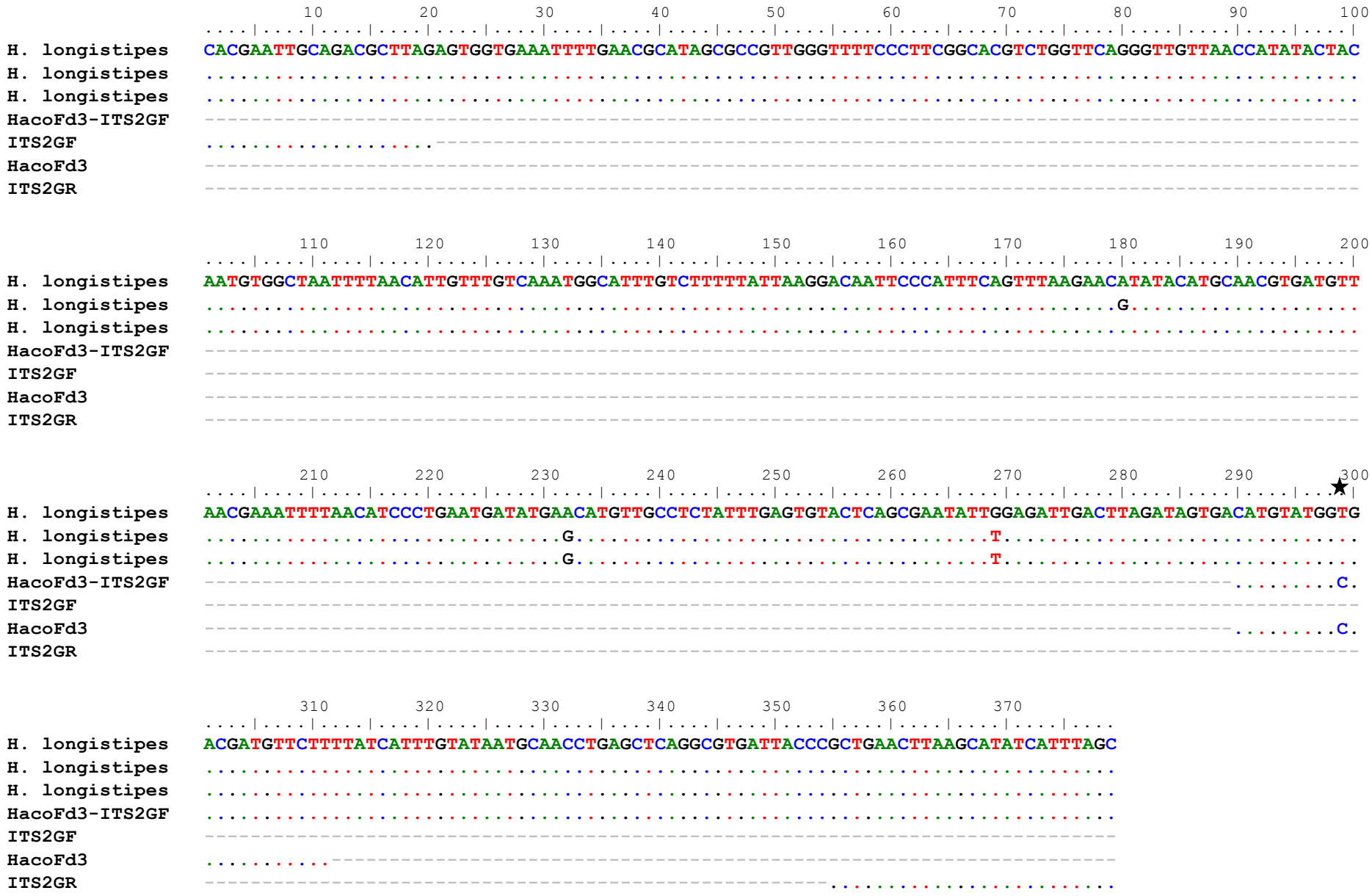

Figure S2 Alignment of the *Haemonchus longistipes* haplotype sequences obtained in this study (accession numbers: MK936878–MK936880, 379 bp) and amplified product from the primer pair HacoFd3 - ITS-2 GR (MK936881, 90 bp) and the three primers ITS-2 GF, ITS-2 GR and HacoFd3. Asterisk indicates the nucleotide polymorphism site between the target sequence and the primer-amplified products.

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