

Morphological and molecular characteristics of *Sarcocystis* sp. in donkeys from China

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Abstract

There is considerable confusion concerning the relationships among species of *Sarcocystis* found in donkeys and horses. Here, sarcocysts obtained from donkeys (*Equus asinus*) were morphologically and molecularly characterized. Sarcocysts were diagnosed in 12 of 32 (37.5%) Chinese donkeys and could be divided into two types, thin-walled and thick-walled, with the aid of light microscopy (LM). The thin-walled sarcocysts were macroscopic (up to 4856 × 320 μm in size) and had short club-like protrusions of up to 2.7 μm; the thick-walled sarcocysts were microscopic (up to 3750 × 135 μm in size) and had villar protrusions of up to 5.4 μm. The ultrastructures of the two types exhibited highly similar morphological characteristics, including bundled microtubules in the core of the villar protrusions penetrating diagonally into the ground substance, similar to wall type 11c. Three genetic markers, 18S rDNA, 28S rDNA, and mitochondrial *cox1*, were sequenced and analyzed in the two types. The three genetic markers presented high intraspecific similarity between the two types, ranging from 97.2–99.5%, 97.8–99.6% and 99.0–99.9%, respectively. The comparison of these sequences with those of *Sarcocystis* spp. previously deposited in GenBank showed that the newly obtained sequences of 18S rDNA, 28S rDNA, and mitochondrial *cox1* presented identities of 90.0–97.5%, 94.7–95.1% and 82.6–84.5%, respectively, with those of *S. bertrami* and *S. fayeri* obtained from horses. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) based on the mitochondrial *cox1* sequences of donkey and horse sarcocysts revealed three fragments (196, 243 and 641 bp) and two fragments (416 and 644 bp), respectively, upon digestion with the restriction enzymes EcoRI and HinfI. Phylogenetic analysis based on 18S rDNA or mitochondrial *cox1* sequences revealed that donkey sarcocysts formed an individual clade with *Sarcocystis* spp. obtained from horses. Based on the divergence of the three sarcocyst genetic markers, especially mitochondrial *cox1*, between donkeys and horses, the *Sarcocystis* sp. sarcocysts in donkeys were different with those obtained from horses. The cross-infection of *Sarcocystis* between donkeys and horses needs to be attempted for further confirmation in the future.

Introduction

The sarcocysts infecting donkeys were first described and named *Sarcocystis asinus* by Gadaev (1978) only based on the size, shape and staining of bradyzoites in smears. Subsequently, on the basis of cross-infection between horses and donkeys and the morphological similarity of the sarcocysts found in the two hosts, *S. asinus* was questioned, and the species of *Sarcocystis* infecting the two hosts came to be regarded as the same species (Levine and Tadros 1980; Odening et al. 1995; Odening 1998). Thus, the name *S. bertrami* is currently used for the sarcocysts diagnosed in the muscles of donkeys by most authors (Dubey et al. 2016; Passantino et al. 2019).

Currently, the sarcocysts found in horses are divided into two forms by Dubey et al. (2015): a macroscopic form (up to 15 mm long) with a thin cyst wall, named *S. bertrami* (synonym *S. equicanis*), and a microscopic form (up to 990 μm long) with a thick cyst wall, named *S. fayeri*. Ultrastructurally, the protrusions of *S. bertrami* sarcocysts are folded on the cyst wall, similar to type 11, but those of *S. fayeri* sarcocysts appear sloping to straight, similar to type 11a. The morphological characteristics of horse

sarcocysts have been observed to undergo changes in various stages of development, showing long protrusions in the early phase and short protrusions in the later phase (Fayer et al. 1983). Therefore, there is still considerable confusion concerning the relationship between *S. bertrami/equicanis* and *S. fayeri* (Odening et al. 1995; Ma et al. 2020).

In recent decades, molecular analysis based on nucleotide sequences has been recommended as a useful and efficient tool for delineating or identifying species of *Sarcocystis* from the same or different hosts. There are currently only limited donkey sarcocyst 18S rDNA and mitochondrial *cox1* sequences deposited in GenBank, which were provided by Zeng et al. (2018). Based on the high similarity of mitochondrial *cox1* sequences, these authors proposed *S. bertrami* (syn. *S. fayeri*) as the descriptor of the parasites of both horses and donkeys. However, the reliability of the meat samples used in their study was based mainly on the applied version of meat cutter (according to communication with the corresponding author of this paper, Dr. Yang). However, in local meat markets, meat sellers or butchers sometimes mix horse and donkey meat or replace donkey meat with horse meat to seek better returns because the price of donkey meat is higher than that of horse meat.

The morphological and molecular characteristics of *S. bertrami* sarcocysts isolated from horses in China have been investigated by our group previously (Ma et al. 2020). To clarify the relationship of the *Sarcocystis* species of donkeys and horses based on the molecular identification of meat samples, the aims of the present study were to investigate the morphological characteristics of donkey sarcocysts and to explore the relationship of the *Sarcocystis* species of donkeys and horses via polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and phylogenetic analysis.

Materials And Methods

Collection and identification of meat samples

Donkey meat serves as a food source for humans and is commonly marketed in China. Muscle tissues from a total of 32 donkeys were purchased in a meat market in Kunming City located in Yunnan Province, China, in October 2019. Fresh muscle tissue (200 g each) from each donkey was examined for sarcocysts.

To further confirm the identity of the meat samples, donkey meat and horse meat (collected previously and stored at -40 °C) infected with sarcocysts were processed for molecular identification in the laboratory. DNA from each animal was extracted using a TIANamp Genomic DNA Kit (TIANGEN BIOTECH CO., LTD, Beijing, China) according to the manufacturer's instructions. Mitochondrial *cox1* was amplified with the primer pair ML1F/ML1R (5'-ACCACAAAGACATCGGCACT-3'/5'-CGTTTGGATGCGAATGCT-3'), designed using Oligo 5.0 software (National Biosciences, Inc., Plymouth, MN, USA) based on the highly conserved areas of mitochondrial *cox1* sequences of *Equus* spp. deposited in GenBank. PCR assays were carried out in 15 µl reaction with 50–100 ng of total DNA, 1 unit of Taq polymerase (TaKaRa, Dalian, China), and 20 pmol of each primer. The amplification program consisted of 35 cycles of 95

°C for 5 min, 54 °C for 30 sec, and 72 °C for 5 min. The PCR products were sequenced on an ABI 3730XL automatic DNA sequencer (Applied Biosystems, Inc., Foster City, California).

Morphological examination of sarcocysts in donkeys

In the laboratory, 10 specimens of approximately 10 × 3 mm in size from each collected sample were pressed and squeezed between two glass slides and then inspected using a stereomicroscope. Thereafter, individual sarcocysts were extracted and isolated from skeletal muscular fibers using needles and processed for light microscopy (LM), transmission electron microscopy (TEM) and DNA analysis.

For TEM, four sarcocysts of each type were fixed in 2.5% glutaraldehyde in cacodylate buffer (0.1 M, pH 7.4) at 4 °C, postfixed in 1.0% osmium tetroxide in the same buffer, dehydrated in a graded alcohol series, and embedded in an Epon-Araldite mixture. Ultrathin sections were stained with uranyl acetate and lead citrate and then examined using a JEM100-CX transmission electron microscope (JEOL Ltd., Tokyo, Japan) at 100 kV.

Molecular characterization

For DNA analysis, three individual sarcocysts of each type obtained from donkey meat and one sarcocyst isolated from identified molecularly horse meat were subjected to genomic DNA extraction using a TIANamp Genomic DNA Kit. Three genetic markers, 18S rDNA, 28S rDNA and mitochondrial *cox1*, were amplified from donkey sarcocysts with the primer sets S1/S4 (Fischer and Odening 1998), KL1/KL3, KL4/KL5b and KL6a/KL2 (Mugridge et al. 1999), and SF1/SR9 (Gjerde 2013, 2014), respectively. PCR products were gel purified, cloned, and sequenced using the methods detailed in a previous paper (Hu et al. 2016). Only mitochondrial *cox1* was amplified from the sarcocyst obtained from horse meat using the SF1/SR9 primers for RFLP. To avoid increasing uncertainties, the 18S rDNA and mitochondrial *cox1* sequences of donkey sarcocysts provided by Zeng et al. (2018) were not used in the present analysis.

To establish the PCR-RFLP strategy for discriminating the sarcocysts of donkeys and horses, the internal endonuclease cleavage sites in the mitochondrial *cox1* nucleotide sequences of the sarcocysts of the two hosts were screened using Premier 5.0 software (Premier, Canada). Three mitochondrial *cox1* nucleotide sequences (MH025631–MH025633) of *S. bertrami* from horses morphologically identified in our laboratory (Ma et al. 2020) were used in the analysis of endonuclease cleavage sites. Based on the screening results, two restriction enzymes, EcoRI and HinfI, were selected to digest the amplified PCR products of mitochondrial *cox1* from the sarcocysts of the two hosts because of their ability to produce different numbers of fragments from the sarcocysts of donkey (three, of 196, 243 and 641 bp) and horse (two, of 416 and 644 bp). Aliquots of 10 µl of the resulting PCR products were double digested in 20 µl reactions with 1 µl EcoRI and 1 µl HinfI and 10 × Cutsmart buffer following the recommendations of the manufacturer (New England BioLabs). The digested products were analyzed via gel electrophoresis on 2% agarose gels stained with Goldenvue at 100V in 0.5× TBE buffer.

Phylogenetic analyses were conducted separately on the nucleotide sequences of the 18S rDNA and mitochondrial *cox1* sequences by using MEGAX software (Kumar et al. 2018). The maximum likelihood (ML) trees of 18S rDNA and mitochondrial *cox1* were generated with the Hasegawa-Kishino-Yano and Kimura 2-parameter models, respectively, according to the Find Best DNA/Protein Models program integrated into MEGAX. The reliability of the maximum likelihood phylograms was tested via the bootstrap method using 1000 replications.

The 18S rDNA and mitochondrial *cox1* sequences of *Sarcocystis* spp. from different hosts were downloaded from GenBank and aligned using the ClustalW program implemented in MEGAX. The final alignment of the 18S rDNA sequences consisted of 31 nucleotide sequences and 1046 aligned positions from 24 taxa. *Cystoisospora ohioensis* (GU292304), *Hammondia hammondi* (KF854253), and *Toxoplasma gondii* (U12138) were chosen as outgroups. The final alignment of mitochondrial *cox1* sequences consisted of a total of 27 nucleotide sequences and 933 aligned positions from 20 taxa. *Hammondia triffittae* (JX473247), *Hammondia heydorni* (JX473251) and *T. gondii* (JX473253), were used as outgroup species to root the tree.

Results

Molecular identification of meat samples

The 14 meat samples (12 for donkeys and 2 for horses) infected with sarcocysts were molecularly identified based on mitochondrial *cox1* sequences. The 12 newly obtained mitochondrial *cox1* sequences from donkeys were 1408 bp long and shared 99.7–100% identity (average 99.9%); the two newly obtained mitochondrial *cox1* sequences from horses were 1408 bp and shared 100% identity. Therefore, only three nucleotide sequences from donkeys and one nucleotide sequence from the horse were deposited in GenBank, with accession numbers ON459761– ON459763, and ON459764, respectively. The identity between the sequences of donkeys and horses was 92.1–92.4% (average 92.3%). At this locus, the newly obtained nucleotide sequences from donkey and horse shared 97.8–100% (average 99.3%) and 98.2–100% (average 99.4%) identity, respectively, with those of *E. asinus* and *E. caballus* previously deposited in GenBank.

Morphological observation of sarcocysts in donkeys

Sarcocysts were found in 12 of 32 (37.5%) donkeys. Using LM, the sarcocysts could be divided into two types: thin-walled and thick-walled (Fig. 1a, c). The thin-walled sarcocysts were macroscopic, measuring 2350–4856 × 110–320 µm [average = 2787 (± 442) × 210 (±64) µm, (±SD); *n* = 20 isolated from five donkeys] in size, and exhibited numerous short club-like protrusions with lengths of 2.0 to 2.7 µm (mean = 2.4 ± 0.18 µm; *n* = 20 measurements from 10 sarcocysts); they were septate and contained bradyzoites measuring 14.5–17.4 × 3.5–5.0 µm [average = 15.8 (±1.8) × 4.2 (±0.4) µm, *n* = 20 measurements from three sarcocysts] in size. The thick-walled sarcocysts were microscopic, measuring 1200–3750 × 45–135 µm [average = 2213 (± 126) × 98 (±21) µm, *n* = 20 isolated from four donkeys] in size and showed villar protrusions with lengths of 3.0 to 5.4 µm [mean = 4.2 (±0.25) µm, *n* = 20 measurements from 10

sarcocysts]; they were septate and contained bradyzoites measuring $12.1-16.2 \times 2.5-4.7 \mu\text{m}$ [average = $14.5 (\pm 1.2) \times 4.1 (\pm 0.3) \mu\text{m}$, $n = 20$ measurements from five sarcocysts] in size.

By TEM, the macro and micro sarcocysts exhibited similar morphological characteristics (Fig. 1 b, d): the primary cyst wall had numerous villar protrusions with bundled microtubules in the core, which penetrated diagonally into the ground substances and sometimes reached the interior border of the ground substance. Minute undulations were present over the entire sarcocyst surface. A layer of ground substance was present beneath the protrusions. Overall, the cyst wall was similar to TEM type 11 classified by Dubey et al. (2015). Additionally, the tilt of protrusions of the two types of sarcocysts were different. The protrusions of thin-walled sarcocysts were almost folded over the cyst wall (similar to TEM subtype 11c), but those of thick-walled sarcocysts were sloping on the cyst wall (similar to TEM subtype 11a).

Molecular characterization of sarcocysts in donkeys

The three selected genes (18S rDNA, 28S rDNA and mitochondrial *cox1*) were successfully amplified from six individual sarcocysts (three thin-walled cysts and three thick-wall cysts) isolated from four donkeys. The three 18S rDNA sequences (accession numbers OM971696–OM971698) of the thin-walled sarcocysts were 1591–1614 bp long and shared 97.7–99.8% identity (average 98.4%). The three 18S rDNA sequences (OM971699–OM971701) of the thick-walled sarcocysts were 1589–1607 bp long and shared 97.7–98.5% identity (average 98.2%). The similarity between the two types was 97.2–99.5% (average 97.8%). The most similar sequences in GenBank to the newly obtained 18S rDNA sequences were those of *Sarcocystis* spp. obtained from horses in different regions, including *S.*

bertrami (MH025625–MH025628) from Chinese horses (95.6–97.5% identity, average 96.5%), *S. fayeri* (LC171838) from an Italian horse (95.2–97.1%, average 96.3%), *S. fayeri* (AB661437–AB661447) from Japanese horses (90.8–97.4% identity, average 94.7%), *S. fayeri* (AB972440–AB972443 and LC171831–LC171837) from Canadian horses (90.0–97.1% identity, average 94.1%), and *S. fayeri* (MF614956) from an Egyptian horse (93.7–93.8%, average 93.8%).

The three 28S rDNA sequences (OM971683–OM971685) obtained from thin-walled sarcocysts were 3441–3450 bp length and shared 97.7–98.5 identity (average 98.1%). Only two 28S rDNA sequences (OM971686 and OM971687) of thick-walled sarcocysts were successfully assembled. They were 3445 and 3446 bp in length and shared 98.8% identity. The similarity between the two types was 97.8–99.6% (average 98.4%). The most similar sequences were those of *S. bertrami* (MH025629–MH025630) from Chinese horses (94.7–95.1% identity, average 94.9%), followed by those of *S. sui hominis* (MK867471–MK867473) obtained from domestic pigs (90.0–91.2% identity, average 90.7%).

The three mitochondrial *cox1* sequences (OM970235–OM970237) of thin-walled sarcocysts were 1085 bp in length and shared 99.2–99.7 identity (average 99.4%). The three mitochondrial *cox1* sequences (OM970238–OM970240) of thick-walled sarcocysts were 1085 bp in length and shared 99.2–99.3% identity (average 99.3%). The identity between the two types was 99.0–99.9% (average 99.4%). The most similar sequences in GenBank was those of *Sarcocystis* spp. obtained from horses in different regions,

including *S. fayeri* (LC171840–LC171854) from Canadian horses (82.8–84.5% identity, average 83.9%), *S. bertrami* (MH025631–MH025633) from Chinese horses (82.3–83.2% identity, average 82.9%), *S. fayeri* (LC171857) from an Italian horse (83.1–83.8% identity, average 83.4%), and *S. fayeri* (LC171855 and LC171856) from Japanese horses (82.7–83.4% identity, average 83.1%).

PCR-RFLP based on mitochondrial *cox1* obtained from donkey and horse sarcocysts

The PCR-amplified products of mitochondrial *cox1* from donkey and horse sarcocysts were successfully digested by EcoRI and HinfI. This produced three fragments (196, 243 and 641 bp) and two fragments (416 and 644 bp) for donkey and horse, respectively (Fig. 2).

Phylogenetic analysis

Phylogenetic analysis based on the newly obtained 18S rDNA and mitochondrial *cox1* sequences confirmed their association with *Sarcocystis* species, and *Sarcocystis* sp. obtained from donkeys formed an individual clade with *S. bertrami* and *S. fayeri* obtained from horses in different areas (Fig. 3, 4).

Discussion

Sarcocystis is a common parasitic protozoan with a worldwide distribution found in a variety of mammals and birds, especially in domesticated food animals. Sarcocysts have been diagnosed in donkeys from the former USSR (Gadaev 1978), Austria (Hinaidy and Loupal 1982), Germany (Matuschka 1983), Morocco (Kirmse 1986), Egypt (Hilali and Nasser 1987; Dubey et al. 2016), China (Hu et al. 2001), and Italy (Passantino et al. 2019). In the present study, the prevalence of sarcocysts in the investigated Chinese donkeys was 37.5% (12/32), and it has been reported to be higher than 22.0% (9/41) in Moroccan donkeys (Kirmse 1986) and 28.6% (40/140) in Italian donkeys (Passantino et al. 2019), but lower than 40% (8/20) in the former USSR (Gadaev 1978), 90.0% (18/20) in Egyptian donkeys (Hilali and Nasser 1987) and 92.3% (24/26) in Chinese donkeys surveyed by our group 20 years ago (Hu et al. 2001).

In the present study, two types of sarcocysts (thin-walled cysts and thick-walled cysts) were observed in the muscle tissues of donkeys under LM. The thin-walled sarcocysts were macroscopic (up to 4856 long and 320 µm wide) and had short club-like protrusions (up to 2.7 µm long); the thick-walled sarcocysts were microscopic (up to 3750 µm long and 135 µm wide) and had villar protrusions (up to 5.4 µm long). The two types of sarcocysts were probably associated with the length of development. Fayer et al. (1983) experimentally infected ponies with sporocysts collected from dogs that had been fed horsemeat containing visible sarcocysts. On Day 127 post-infection (PI), the sarcocysts were still immature (metrocysts) and measured 12.6–30 × 50–360 µm. some walls had long protrusions (4.5 µm), and others showed short protrusions (1 to 2 µm). However, on Days 157 and 184 PI, all sarcocysts were mature (bradyzoites) and up to 436.6 µm long. They only had short protrusions. Matuschka et al. (1986) performed a similar experimental infection and observed microscopic sarcocysts (< 1 mm long) and

macroscopic sarcocysts (up to 2 mm) in ponies on Day 378 PI. However, on Day 1040 PI, only macroscopic sarcocysts were found, with sizes of up to 9 × 0.5 mm.

The ultrastructure of the sarcocyst wall is useful in evaluating the taxonomy of *Sarcocystis* species in a given host. Dubey et al. (2015) grouped sarcocysts into at least 42 types and several subtypes based on TEM morphological characteristic of sarcocyst wall. In our materials, the ultrastructures of the thin-walled and thick-walled sarcocysts presented characteristics of TEM type 11: the protrusions of the sarcocyst wall contained bundled microtubules in the core of the protrusions penetrated into the ground substance. Based on the inclination of protrusions over the sarcocyst surface, the TEM types of thin-walled and thick-walled sarcocysts could be subdivided into TEM type 11c and 11a. All ultrastructural descriptions of sarcocysts obtained from donkeys provided by different authors to date conform to the characteristics of TEM type 11 (Hilaili and Nasser 1987; Dubey et al. 2016; Passantino et al. 2019). Among them, sarcocysts from Egyptian donkeys are similar to TEM type 11c (Hilaili and Nasser 1987, Dubey et al. 2016), and those from Italian donkeys are similar to TEM type 11a (Passantino et al. 2019). These sarcocysts were named as *Sarcocystis* sp. (Hilali and Nasser, 1987), or *S. bertrami* (Dubey et al. 2016, Passantino et al. 2019).

Morphologically similar sarcocysts frequently occur in different hosts, especially in closely related hosts, as observed in the sibling species *S. tenella* and *S. capracanis* found in sheep and goats, respectively, which sometimes creates controversy regarding species identification (Formisano et al. 2012; Dubey and Rosenthal 2013). Currently, PCR assays and sequencing procedures are considered much more practical, accurate, and reliable for the delineation and identification of *Sarcocystis* species than traditional methods based on morphological characteristics (Gjerde 2013). Therefore, a critical comparison of the molecular characteristics of *Sarcocystis* species in donkeys and horses should be performed to help reach a final conclusion (Dubey et al. 2016).

In the present study, three genetic markers, 18S rDNA, 28S rDNA, and mitochondrial *cox1*, were sequenced and analyzed in the two types of sarcocysts found in donkeys. The sequences of the three loci in the two types presented high intraspecific similarities of 97.2 – 99.5% (on average 97.8%), 97.8 – 99.6% (on average 98.4%) and 99.0 – 99.9% (on 99.4%), respectively. Therefore, combined with the similar morphological features observed under TEM, the two types of sarcocysts observed in donkeys are inferred to represent a same *Sarcocystis* species. The comparison of the newly obtained 18S rDNA, 28S rDNA and mitochondrial *cox1* sequences with those deposited in GenBank showed identities of 90.0 – 97.5% (average 94.7%), 94.7 – 95.1% (average 94.9%) and 82.6 – 84.5% (average 83.4%), respectively, with those of *S. bertrami* and *S. fayeri* obtained from horses. Additionally, the difference between the donkey and horse sarcocysts was proven by PCR-RFLP based on the mitochondrial *cox1* sequences of the two parasites. Therefore, the sarcocysts of donkeys should not belong to the species *S. bertrami* or *S. fayeri* found in horses.

Cross-infection is a criterion for revealing whether different intermediate hosts harbor the same parasite. To date, there has been only one reported attempt to perform the cross-infection of *Sarcocystis* between

donkey and horse (Matuschka 1983). Tissues from 20 horses naturally infected with sarcocysts were fed to a dog, and those of 10 donkeys were fed to another dog. Both dogs excreted sporocysts. Experimental infections were carried out in 4 ponies (#1–4). One pony (#1) fed donkey-derived sporocysts became febrile on Days 10 and 11 and 19 – 21, but no sarcocysts were detected on Days 44 and 59 PI. The same pony was then fed horse-derived sporocysts on Day 117 and killed on Day 138 PI. Only immature sarcocysts were identified in the carcass. The other three ponies (#2 – 4) were fed horse-derived sporocysts and killed on Days 197, 212, and 21 PI, respectively. Mature sarcocysts were detected in ponies #2 and 3, but no sarcocysts were found in pony #4. These results suggest the possible transmission of the parasite in horses and donkeys. However, Fayer and Dubey (1982) found immature sarcocysts ($9.2 \times 14.4 \mu\text{m}$ in sizes) on Day 55 PI and mature sarcocysts ($35.6 \times 81.8 \mu\text{m}$ in sizes) on Day 77 PI in horses infected with horse-derived sporocysts. Therefore, the experiments performed by Matuschka (1983) are insufficient to support the cross-infection of *Sarcocystis* between donkey and horse for reasons including the following: (1) transmission was based only on one pony (#1) fed donkey-derived sporocysts; (2) no mature sarcocysts (on Day 138 PI) were detected in the experimental donkey; and (3) immature sarcocysts (on Day 138 PI) were detected in the experimental donkey, however this animal was infected twice, first with donkey-derived sporocysts and second with horse-derived sporocysts (on Day 117 PI). Therefore, the cross-infection of *Sarcocystis* between donkey and horse should be attempted, and the available molecular evidence also needs to be supplemented in the future.

In summary, two types of sarcocysts were observed in donkeys in China. Based on morphological traits and analysis of the three genetic markers, the two types are attributed to one parasite. Compared the newly obtained sequences with those of *S. bertrami* and *S. fayeri* in horses previously deposited in GenBank, the parasite in donkeys are distinct from *S. bertrami* and *S. fayeri* in horses. Gadaev (1978) first proposed the name *S. asinus* for the sarcocysts found in donkeys, but few cyst morphological characteristics and no molecular data provided. Considering the arguments of *Sarcocystis* spp. in horse and other equids (Dubey et al., 2015), here, *Sarcocystis* sp. was used to name the sarcocysts found in donkeys to avoid increasing uncertainty.

Declarations

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Conflicts of interest/Competing interests

The authors declare that they have no conflicts of interest.

Availability of data and material

No other data and material are provided

Authors' contributions

JH suggested the overall concept and design of the study, and drafted the manuscript. MZ, KW, and ZW, conducted specimen collection and molecular work. JS and SD performed observation of sarcocysts and data analysis. JT provided suggestions for this manuscript. All authors reviewed the manuscript.

Ethics approval

The present study was approved by the Animal Ethics Committee of Yunnan University (permission number AECYU2018004)

Consent to participate

The authors declare that they have participated this work.

Consent for publication

The authors declare that they know the content of this manuscript and agree to submit it to Parasitology Research.

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Figures

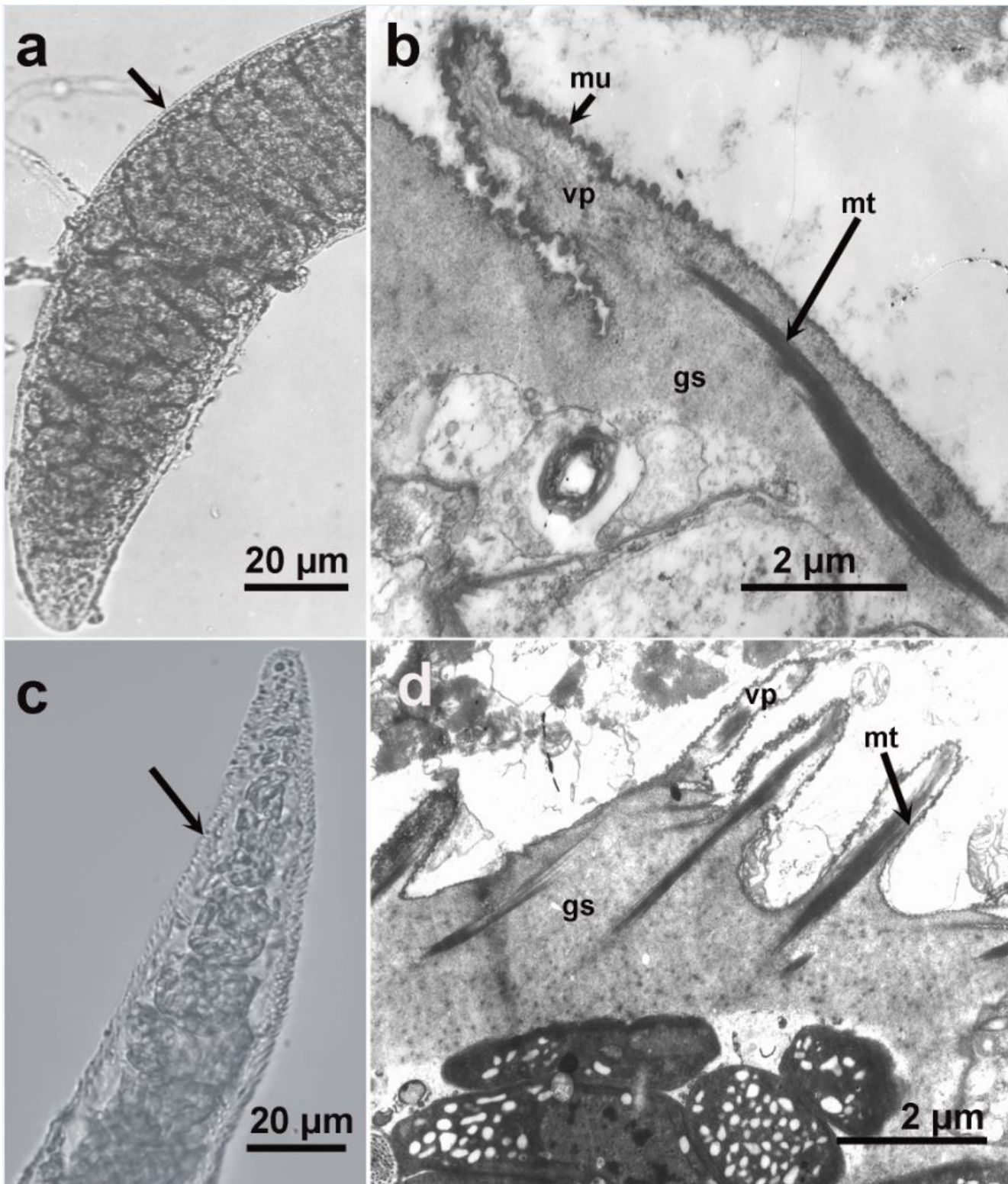


Figure 1

Morphological characteristics of *Sarcocystis* sp. sarcocysts isolated from skeletal muscles of donkeys. **a** Thin-walled sarcocyst (unstained, light microscopy, LM) bound by short club-like protrusions (arrow). **b** Diagonal section of a thin-walled sarcocyst (under transmission electron microscopy, TEM). The sarcocyst wall exhibits numerous villar protrusions (vp), which are often bent along the cyst surface. The vp contain bundled microtubules (mt) in their core, which penetrate diagonally into the ground substance

(gs). Minute undulations (mu) present over the entire sarcocyst surface. **c** Thick-walled sarcocyst (unstained, under LM) bound by villar protrusions (arrow). **d** Longitudinal section of a thick-walled sarcocyst (under TEM). The sarcocyst wall exhibits numerous vp, which are often sloping on the cyst surface. The vp contain bundled microtubules (mt) in the core, which penetrate diagonally into the ground substance (gs).

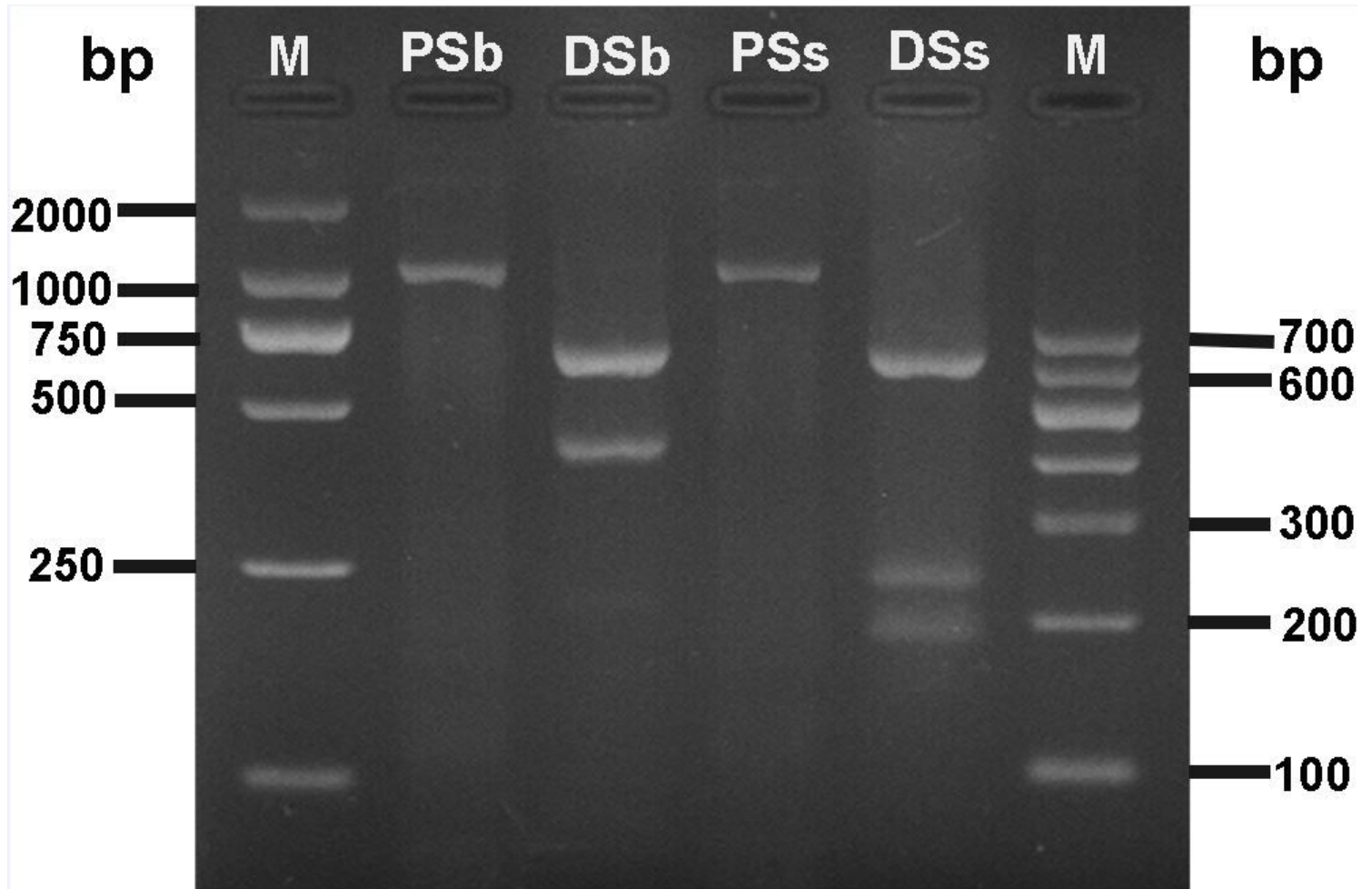


Figure 2

Results of PCR with primers SF1/SR9 and restriction enzyme digestion with EcoRI and HinfI and for sarcocyst DNA from *S. bertrami* from a naturally infected horse and *Sarcocystis* sp. isolated from a naturally infected donkey. M, molecular mass marker; PSb, PCR product of *S. bertrami*; DSb, digestion of PCR product of *S. bertrami* with EcoRI and HinfI; PSs, PCR product of *Sarcocystis* sp.; DSs, digestion of PCR product of *Sarcocystis* sp. with EcoRI and HinfI.

Figure 3

Phylogenetic tree based on 18S rDNA sequences. The tree was built using the maximum likelihood (ML) with the Hasegawa-Kishino-Yano model. The analysis involved 31 nucleotide sequences (GenBank

accession numbers behind the taxon names) and a total of 1046 aligned positions in the final dataset. The values between the branches represent bootstrap values per 1000 replicates, and values below 50% are not shown. The six new sequences of *Sarcocystis* sp. (OM971696–OM971701, shown in boldface) in donkeys formed an individual clade with *S. bertrami* and *S. fayeri* in horses.

Figure 4

Phylogenetic tree based on mitochondrial *cox1* sequences. The tree was built using the maximum likelihood (ML) with the Kimura 2-parameter model. The analysis involved 27 nucleotide sequences (GenBank accession numbers behind the taxon names) and a total of 933 aligned positions in the final dataset. The values between the branches represent bootstrap values per 1000 replicates, and values below 50% are not shown. The six new sequences of *Sarcocystis* sp. (OM970235–OM970240, shown in boldface) obtained from donkeys formed an individual clade with *S. bertrami* and *S. fayeri* obtained from horses.