

# *Sarcocystis mehlhorni*, n. sp. (Apicomplexa: Sarcocystidae) from the black-tailed deer (*Odocoileus hemionus columbianus*)

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**Abstract** Infection with *Sarcocystis* is common in many species of wild cervids but none is reported from the black-tailed deer (*Odocoileus hemionus columbianus*). Here, we report *Sarcocystis* infection in two black-tailed deer from northwest USA for the first time. Sarcocysts were microscopic, up to 556 µm long and mature. The sarcocyst wall was up to 1.39 µm thick and had rectangular 1.17-µm-long villar protrusions, type 17, with thin (230 nm) electron dense ground substance layer. Molecular characterization and phylogenetic analysis indicated that *Sarcocystis* in the black-tailed deer is related to structurally distinct *Sarcocystis* species in cervids. A new name, *Sarcocystis mehlhorni*, is proposed for the *Sarcocystis* species in black-tailed deer.

**Keywords** Black-tailed deer · *Odocoileus hemionus columbianus* · *Sarcocystis* · Ultrastructure · Phylogeny

## Introduction

Infection with *Sarcocystis* is common in many species of wild cervids, but none is reported from the black-tailed deer

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(*Odocoileus hemionus columbianus*) (Dubey et al. 2015a). Here, we report *Sarcocystis* infection in two black-tailed deer from northwest USA for the first time.

## Material and methods

Tongue and myocardium were collected from two (#14183, #15030) adult black-tailed deer (*O. hemionus columbianus*) from Pierce County, WA, USA, on 11 December 2014 and 19 April 2015. Animals were lethally removed by US Department of Agriculture, Animal and Plant Health Inspection Service personnel during an authorized direct control project. Samples were immediately collected. Refrigerated tongues and hearts were shipped (overnight delivery) to the Animal Parasitic Diseases Laboratory, USDA, Beltsville, Maryland, the following day, for testing for protozoal infections.

## Microscopical examination

A portion of tissues was fixed in buffered formalin and processed for histology using hematoxylin and eosin (HE) stain of 5-µm-thick sections. The number of cysts per section (2 × 0.7 cm) was recorded. Fresh muscles were examined for sarcocyst presence by squeezing; individual cysts were excised and kept frozen until molecular assays. Five cysts detected in the HE sections of deer #14183 were excised and processed for transmission electron microscopy (TEM) as reported by Dubey et al. (2015b).

## Molecular analyses

Thirteen morphologically similar individual cysts were excised from tissues in deer #15030 and subjected to DNA isolation using DNeasy Blood and Tissue Kit (Qiagen Inc.,

Valencia, CA, USA) according to manufacturer's instructions. DNA quantification and quality were determined by Thermo Scientific NanoDrop Lite Spectrophotometer (Thermo Scientific, Waltham, MA, USA).

The DNA was characterized by PCR amplification and sequencing of two regions of the nuclear ribosomal DNA unit, *18S rRNA* and *28S rRNA*, and the mitochondrial cytochrome c oxidase subunit 1 (*cox1*) locus. The complete regions of *18S rRNA* and *28S rRNA* were amplified using overlapping fragments and primer pairs; ERIB1/S2r, S5f/S4r, S3f/Primer Bsarc, and KL1/LS2R, LS1F/KL3, respectively, as described previously (Gjerde and Josefsen 2015). The partial sequence of *cox1* locus was also amplified using primer pair SF1/SR5 (Gjerde 2013, 2014; Gjerde and Josefsen 2015). The amplified PCR products were run on 2.5 % (w/v) agarose gel.

The single PCR amplicons of *18S rRNA*, *28S rRNA*, and *cox1* were excised from the gel and purified using QIAquick Gel Extraction (Qiagen, Inc., Valencia, CA, USA) according to the manufacturer's recommendations. The purified PCR products were sent to Macrogen Corporation (Rockville, MD, USA) for direct sequencing using the same primer pair used in PCR amplification to obtain both strands reads. The resulting sequences were edited manually if necessary, and analyzed using the software Geneious version 8.0.4 (Biomatters Ltd. Auckland, New Zealand). The sequences obtained were aligned against each other and the published sequences of various *Sarcocystis* spp. to detect intraspecies and interspecies variation on these DNA regions, respectively.

Phylogenetic trees were estimated to show relationship among reference *Sarcocystis* species by the neighbor-joining algorithm applied to Tamura-Nei genetic distances, as implemented by Geneious version 8.0.4. Trees were tested by selecting bootstrap method with a value of 1000 bootstrap replicates. Phylogenetic trees based on *18S rRNA* and *cox1* sequences were constructed using sequences obtained from the black-tailed deer sarcocysts and previously published sequences of various species of *Sarcocystis* in NCBI GenBank.

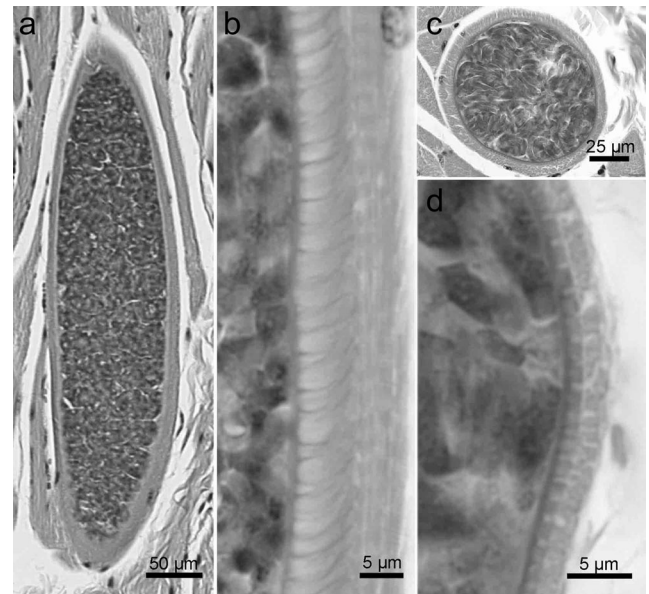
## Results

In wet mounts, sarcocysts were fusiform and up to 600  $\mu\text{m}$  ( $n=13$ ); released bradyzoites from broken cysts were banana-shaped and 12  $\mu\text{m}$  long ( $n=10$ ). Microscopic examination of HE-stained tissue sections revealed 9 cysts in heart and 74 in tongue of deer #14183, and 19 sarcocysts (3 in heart and 16 in tongue) in deer #15030. Only 2 sarcocysts were cut longitudinally and they measured 330 and 556  $\mu\text{m}$  long. In cross-sections, sarcocysts were 36.0–163  $\mu\text{m}$  wide. Under lower magnification, the sarcocyst wall appeared smooth, but at  $\times 1000$  illumination, polygonal or elongated villar protrusions

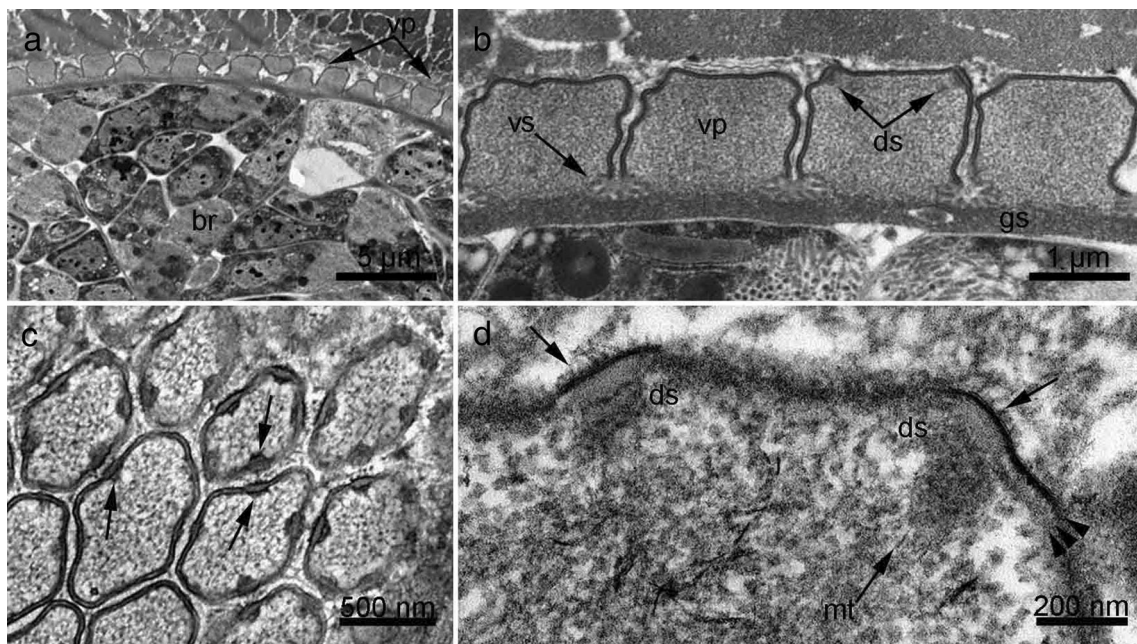
were evident (Fig. 1). Bradyzoites were separated in groups by septa.

One mild focus of myocarditis was observed in deer #15030.

By TEM, the parasitophorous vacuolar membrane was folded into villar protrusions (vp) and lined by a 15–20-nm thick electron dense layer (Fig. 2a, b). Total thickness of primary cyst wall ranged from 29 to 44 nm. The vp were rectangular, type 17 (Dubey et al. 2015a), and measured 1.17  $\mu\text{m}$  (1.50–0.92) long and 1.42  $\mu\text{m}$  (1.98–1.20) wide ( $n=66$ ). The ground substance layer (gs) was remarkably thin (184–278 nm; studied in 4 cysts) and electron dense, that continues as septa (88.0–312 nm wide). Up to 6 disc-shaped plaques were present in the vp (Fig. 2c). Structurally, plaques were 200 nm wide, with at least 3 different layers, numerous microtubules connected them to the core of the vp, and several microfilaments can be seen arising from the parasitophorous vacuolar membrane (Fig. 2d). The total thickness of the sarcocyst wall including the vp and ground substance was 1.39  $\mu\text{m}$  (1.70–1.16) thick. Bradyzoites were 10.4 $\times$ 2.1  $\mu\text{m}$  (8.3–11.5 $\times$ 1.6–2.7;  $n=12$ ) in size and with a prominent mitochondrion, nucleus (nu), numerous micronemes (mn), dense granules (dg), amylopectin granules (am) (Fig. 3a), two rhoptries (rh1, rh2; Fig. 3b), and a conoid (co) with 22 subpellicular tubules and 7 internal tubules (Fig. 3c). Metrocytes, seen in the periphery, were 5.6  $\mu\text{m}$  (5.0–6.0;  $n=4$ ) in size but are not described in detail because they were not well preserved.



**Fig. 1** Light micrographs of *Sarcocystis mehlhorni*, n. sp. from black-tailed deer. **a, b** Longitudinally cut cysts. HE stain. **c, d** Transversally cut cysts. HE stain. **a** Note the septa and the thin wall. **b** Appearance of vp resembling keys of a piano. **c** Thin-walled sarcocyst. **d** Vp with rectangular appearance and remarkable dark ground substance



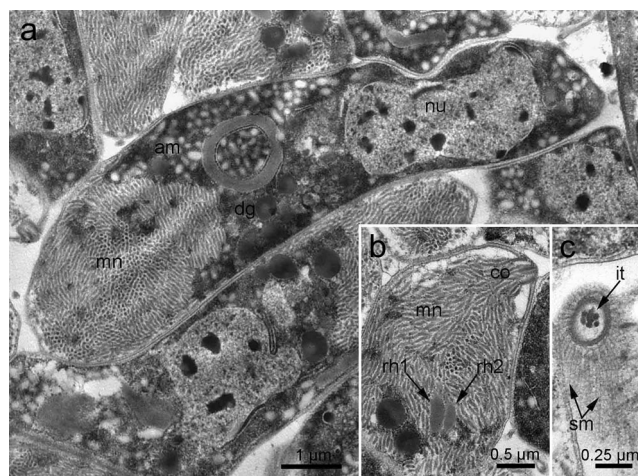
**Fig. 2** TEM micrographs of *Sarcocystis mehlhorni*, n. sp. from black-tailed deer. **a** Sarcocyst with polygonal-shaped villar protrusions (vp) on the wall and enclosed bradyzoites (br). **b** Details of the cyst wall; note rectangular vp with disc-shaped plaques (ds), vesicles (vs), and thin electron dense ground

substance (gs). **c** Cross/oblique section of vp showing several ds (arrows) from cyst #4. **d** Higher magnification of two ds; note the presence of microtubules (mt), three visible layers (arrowheads), and microfilaments (arrows) that arise from the parasitophorous vacuolar membrane

The *18S rRNA* (in three fragments), *28S rRNA* (in two fragments), and *cox1* loci were amplified by PCR using DNA obtained from the individual sarcocysts of black-tailed deer. DNA sequencing of PCR amplicons resulted the unambiguous sequences of two nuclear DNA regions: *18S rRNA* (1819 bp), *28S rRNA* (1598 bp), and the mitochondrial DNA locus, *cox1* (1002 bp, 1022 bp). These

sequences were submitted to NCBI GenBank with accession numbers KT378042 (*18S rRNA*), KT378043 (*28S rRNA*), and KT378044, KT378045 (*cox1*). Phylogenetic analysis based on both the *18S rRNA* and the *cox1* sequences obtained from the DNA of individual sarcocysts of *Sarcocystis mehlhorni* indicated an especially close relationship to another parasite in this genus that employs *Candida* as their definitive host, *Sarcocystis tarandivulpes* (Figs. 4, 5). The *18S rRNA*, *28S rRNA*, and *cox1* sequences shared the highest identity with sequences of *S. tarandivulpes* (99.0 %, EF056012), *Sarcocystis tenella* (94.0 %, AF076899), and *S. tarandivulpes* (92.0 %, KC209718), respectively.

In addition, for *18S rRNA*, similarities were found for other *Sarcocystis* spp. that parasitize Cervidae, to note, *Sarcocystis rangi* (EF056011) from reindeer (*Rangifer tarandus*) or *Sarcocystis taeniata* (KF831278) and *Sarcocystis alceslatrans* (KF831275) from moose (*Alces alces*) (Fig. 4). When studying *cox1*, a genetic relationship was observed with *S. taeniata* (KF831253), *Sarcocystis grueneri* (KC209616), and *S. alceslatrans* (KF831248) (Fig. 5).

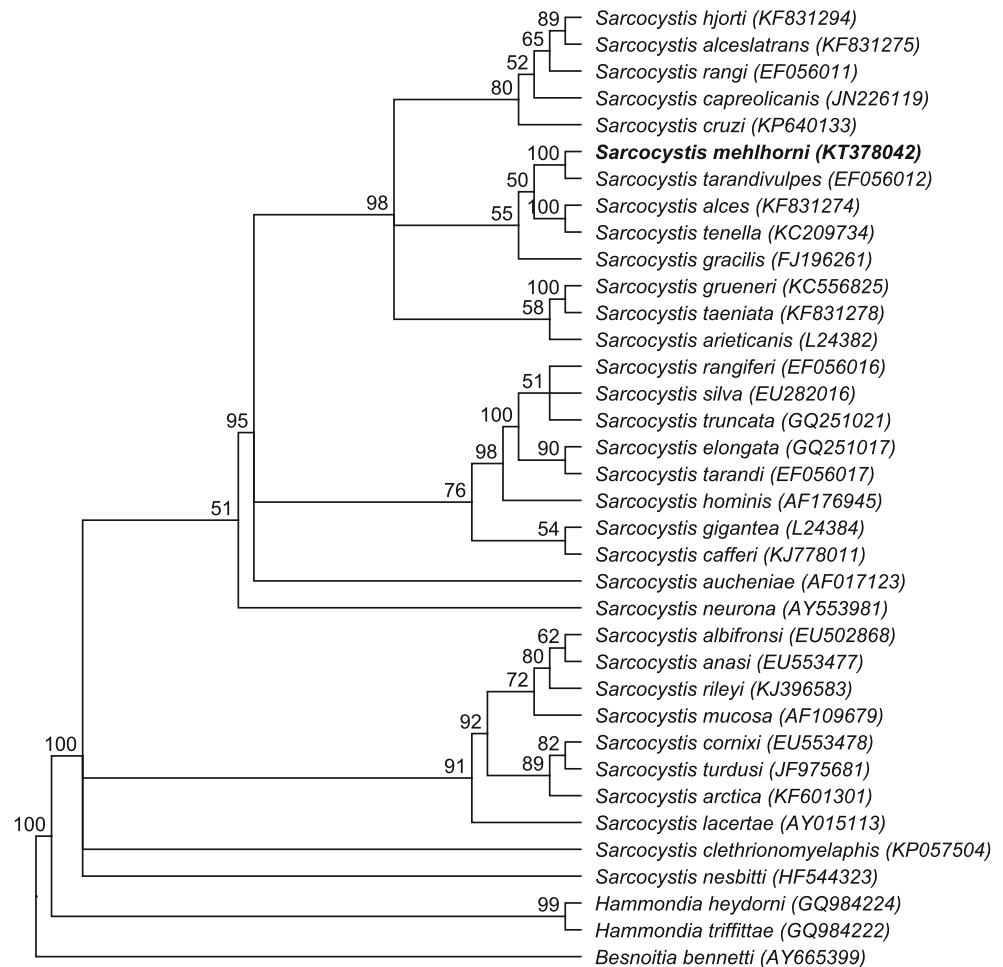


**Fig. 3** TEM micrographs of *Sarcocystis mehlhorni*, n. sp. from black-tailed deer. **a** Longitudinally cut bradyzoite, micronemes (mn), nucleus (nu), amylopectin (am), and dense granules (dg). **b** Conoidal part of a bradyzoite showing two rhoptries (rh1, rh2), conoid (co), and mn. **c** Detail of the co of a bradyzoite; note 7 internal microtubules (it) and 22 subpellicular microtubules (sm)

**Taxonomic summary of *S. mehlhorni* n. sp. (Figs. 1, 2, 3, 4, and 5)**

**Diagnosis** Sarcocysts were microscopic, up to 556 μm long and mature. The sarcocyst wall was up to 1.39 μm thick and had rectangular 1.17-μm-long villar protrusions with up to 6

**Fig. 4** Phylogenetic tree based on *18S rRNA* sequences. Input sequences were the *18S rRNA* regions of various species retrieved from NCBI GenBank and 1819-bp long sequence obtained from *Sarcocystis mehlhorni* sarcocysts from black-tailed deer. Accession number of gene sequence was given in parenthesis following the species name. Tree was built by selecting the Tamura-Nei genetic distance model and neighbor-joining tree methods (Geneious version 8.0.4). Tree was tested by selecting bootstrap method with value of 1,000 replicates. *Sarcocystis mehlhorni* indicated an especially close relationship to other parasites in this genus that employs Canidae as their definitive hosts, *S. tarandivulpes*



disc-shaped plaques, type 17, with thin (230 nm) electron dense ground substance layer.

**Host** Black-tailed deer (*O. hemionus columbianus*).

**Distribution** Washington State (USA), probably other areas in North America.

**Definitive host** Unknown, possibly Canidae.

**Etymology** Species named after Prof. Heinz Mehlhorn who, with Otto Heydorn and Michel Rommel, contributed immensely to our knowledge on biology and structure of *Sarcocystis*.

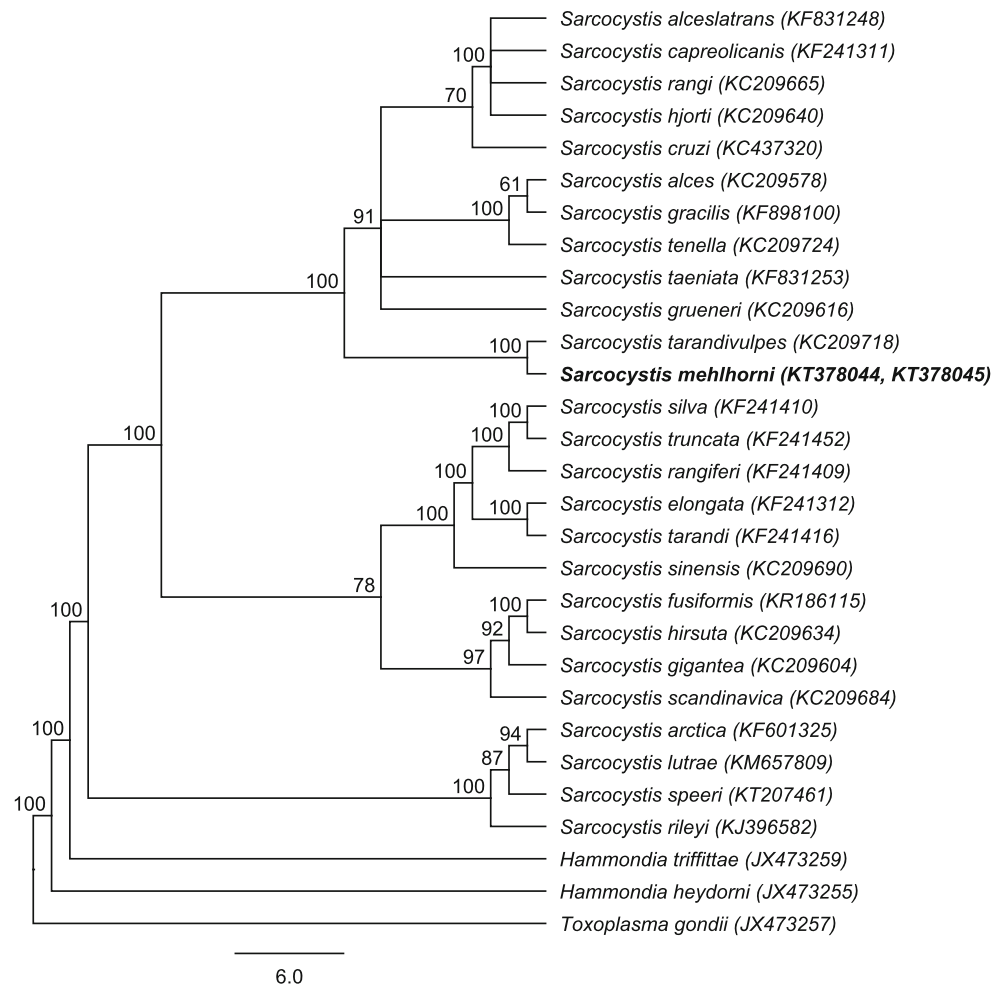
**Specimens deposited** Histological sections stained with H and E and Toluidine blue deposited in the United States National Parasite Collection in the Division of Invertebrate Zoology and National Museum of Natural History, Smithsonian Institution, Washington, D.C. (USNM numbers 1283156 to 128158). Sequences were deposited in NCBI GenBank

accession number KT378042 (*18S rRNA*), KT378043 (*28S rRNA*), and KT378044, KT378045 (*cox1*).

## Discussion

Only one morphological type of sarcocyst was found in the black-tailed deer (BTD) examined and is named *S. mehlhorni*. Its sarcocysts resembled *Sarcocystis odocoileocanis* from the white-tailed deer (WTD) from the USA (Table 1). Crum et al. (1981) named *S. odocoileocanis* for the parasite in WTD from Georgia with canids as the definitive hosts but did not describe its ultrastructure. Entzeroth et al. (1982) first reported the ultrastructure of sarcocysts in WTD from Michigan, USA. The sarcocyst wall was stated to be 15 to 20  $\mu\text{m}$  thick (Entzeroth et al. 1982); however, the authors meant to say 1.5 to 2.0  $\mu\text{m}$  thick (personal communication from B. Chobotar to J. P. Dubey, May 2015). The gs layer was 0.4–1.0  $\mu\text{m}$  thick (Entzeroth et al. 1982); these measurements for the gs in BTD are thinner than in WTD. Otherwise, the descriptions of villar protrusions in WTD and BTD appear similar,

**Fig. 5** Phylogenetic tree based on *cox1* sequences. Input sequences were the *cox1* regions of various species retrieved from NCBI GenBank and sequences of *Sarcocystis mehlhorni* sarcocysts from black-tailed deer (1002 and 1022 bp). Accession numbers of gene sequence were given in parenthesis following the species name. Tree was built by selecting the Tamura-Nei genetic distance model and neighbor-joining tree methods (Geneious version 8.0.4). Tree was tested by selecting bootstrap method with value of 1,000 replicates. *Sarcocystis mehlhorni* clustered together with *Sarcocystis* species that employs Canidae as its definitive hosts, *S. tarandivulpes*



including description of *S. odocoileocanis* from WTD from Florida (Atkinson et al. 1993) and Montana (Dubey and Lozier 1983). One of the prominent feature of the sarcocyst in BTD was the presence of disc-shaped plaques on villar protrusions; these structures were not commented upon but are visible in illustrations provided by Entzeroth et al. (1982), Atkinson et al. (1993), and Dubey and Lozier (1983).

The disc-shaped plaques were first described by Speer and Dubey (1986) in vp of *Sarcocystis hemionilatrantis* in mule deer from the USA. The ultrastructure of *S. odocoileocanis* from the BTD broadly resembles the structure of sarcocysts of *S. hemionilatrantis* in mule deer from the USA and *S. tarandivulpes* from the reindeer in Scandinavia (Gjerde 1984a, 1985, 1986) (Table 1).

For the morphological-related/similar species in wild ruminants, molecular information is only available for *S. tarandivulpes* (Dahlgren and Gjerde 2007; Gjerde 2013). When examining the three markers, molecular similarities were present. Further molecular characterization of *S. odocoileocanis* and *S. hemionilatrantis* will be of interest.

Phylogenetic relationship of *Sarcocystis* sp. from the black-tailed deer with Canidae-transmitted *Sarcocystis* species in wild ruminants suggests that the present species is transmitted by these carnivores.

Transmission experiments are necessary to confirm the final identity of *Sarcocystis* spp. from the black-tailed deer. For example, *S. odocoileocanis* was infective to cattle and sheep (Crum et al. 1981) but not to goat (Lindsay et al. 1988).

Whether the differences on morphological description of parasites in white-tailed deer and mule deer from the USA and *S. tarandivulpes* in reindeer from Norway is related to techniques, description cannot be resolved from the literature.

In conclusion, there is uncertainty concerning the identity of *S. odocoileocanis*-like sarcocysts in cervids. Available morphological and biological data are summarized in Table 1. It is evident that without extensive investigation and transmission experiments (that will not be easily done), the question of species cannot be resolved. To facilitate further studies, we propose a new name, *S. mehlhorni*.

**Table 1** Summary of morphological descriptions provided in the literature for the similar *Sarcocystis* species reported in Cervidae

Species	<i>S. melihorni</i> n. sp.	<i>S. hemionitar-antis</i> <sup>4</sup>	<i>S. odocoileocanis</i> <sup>2</sup>	<i>S. tarandivulpes</i> <sup>4</sup>
Intermediate host	Black-tailed deer ( <i>Odocoileus hemionus columbianus</i> )	Mule deer ( <i>Odocoileus hemionus</i> )	White-tailed deer ( <i>Odocoileus virginianus</i> )	Reindeer ( <i>Rangifer tarandus</i> )
Cyst shape and length	Fusiform, up to 600 µm	ND, up to 525 µm <sup>1</sup>	Spindle-shaped <sup>4</sup> , up to 1449 <sup>1</sup> , 264 <sup>2</sup> , 300–620 <sup>1,3,4</sup>	Spindle-shaped, 0.5–3.0 mm <sup>1,2,3,4</sup>
Primary cyst wall				
Layers	At least 3	4 <sup>5</sup>	ND <sup>1</sup>	2 <sup>5</sup>
Thickness (nm)	29.4–44.4	60.6 <sup>5</sup>	ND <sup>1</sup>	50–60 <sup>5</sup>
Cyst wall				
TEM type (Dubey et al. 2015a)	17	17	17	17
Thickness (µm)	1.16–1.70	1.0–2.0 <sup>2,3</sup>	1.4–2.65 ( <i>n</i> =25) <sup>1</sup> , 2.5–4.6 ( <i>n</i> =17) <sup>3</sup> , 1.5–2.0 <sup>4</sup>	ND <sup>5,6</sup>
Grounds substance				
Thickness (nm)	184–278	ND <sup>2</sup>	400 <sup>1</sup> , 400–600 <sup>3</sup> , 400–1000 <sup>4</sup>	270–550 <sup>5</sup>
Content	Electron dense	Coarsely granular material <sup>2</sup>	Granular material <sup>1</sup> , electron dense <sup>3</sup> , electron lucent <sup>4</sup>	Coarsely granular material <sup>5</sup>
Septa	Present, 88.0–312	Present, 483.8 nm thick <sup>2</sup>	Present <sup>1,2,3,4</sup> , thickness not provided	Present, 160–450 nm thick <sup>1,3,5</sup>
Villar protrusions				
Shape	Rectangular	Inverted T-like <sup>2</sup>	Polygonal (elongated to flat) <sup>1,3</sup>	Polygonal knob-like <sup>1,3,5,6</sup>
Dimensions (µm)	1.17×1.42 (0.92–1.50×1.20–1.98, <i>n</i> =66)	1.92×2.65 (1.78–2.42×1.95–2.78, <i>n</i> =14) <sup>2</sup>	1.9×2.7 (1.9–4.0×1.0–3.2, <i>n</i> =13) <sup>3</sup> , 1.0–1.45×1.18 <sup>4</sup>	0.6–1.2 <sup>5</sup>
Disc-shaped plaques	Present, up to 6	Present, up to 4 <sup>5</sup>	ND <sup>1,3,4</sup>	ND <sup>5</sup>
Vesicles	Present	Present <sup>2,3,4</sup>	Present <sup>2,3,4</sup>	Present <sup>6</sup>
Bradyzoites (µm)	Banana-shaped, 10.4×2.1 (8.3–11.5×1.6–2.7, <i>n</i> =12)	ND <sup>2</sup> , 14.4×2.8 (13.3–15.8×2.6–3.2, <i>n</i> =15) <sup>2</sup>	Banana-shaped, 9.5–14.0×2.8–4.6 ( <i>n</i> =17) <sup>3</sup> , 8.9–12.6×2.12–2.7 <sup>4</sup>	Banana-shaped, 12.6–15.0×3.3–4.8 <sup>3</sup> , 15.0×4.0 <sup>5</sup>
Metrocytes (µm)	5.6 (5.0–6.0, <i>n</i> =4)	7.4×5.3 (5.8–11.0×4.3–6.4, <i>n</i> =12) <sup>2</sup>	Spherical <sup>4</sup> ; 7.6×4.1 (5.0–9.0×2.6–5.5, <i>n</i> =5) <sup>3</sup>	Ovoid, 7.3×4.7 <sup>5</sup>
Sporocysts (µm)	No data	14.4×9.3 ( <i>n</i> =80) <sup>4</sup> ; 16.2×9.9 <sup>6</sup>	15.2×10.7 (13.2–15.7×8.8–12.1, <i>n</i> =195) <sup>2</sup>	13.6×9.8 <sup>4</sup>
Molecular characterization	<i>I</i> 8S, 28S, <i>coxI</i>	No data	No data	<i>I</i> 8S, 28S, <i>coxI</i> <sup>1,2,7</sup>
References	Present paper	<sup>1</sup> Dubey and Lozier (1983), <sup>2</sup> Dubey and Speer (1985), <sup>3</sup> Dubey and Speer (1986), <sup>4</sup> Hudkins and Kistner (1977), <sup>5</sup> Speer and Dubey (1986), <sup>6</sup> Speer et al. (1980)	<sup>1</sup> Atkinson et al. (1993), <sup>2</sup> Crum et al. (1981), <sup>3</sup> Dubey and Lozier (1983), <sup>4</sup> Entzeroth et al. (1982)	<sup>1</sup> Dahlgren and Gjerde (2007), <sup>2</sup> Dahlgren et al. (2007), <sup>3</sup> Gjerde (1984a), <sup>4</sup> Gjerde (1984b), <sup>5</sup> Gjerde (1985), <sup>6</sup> Gjerde (1986), <sup>7</sup> Gjerde (2013)

ND indicates illustrated but not described

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