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Author(s): W. J A. Saville , J. P. Dubey , M. J. Oglesbee , C. D. Sofaly , A. E. Marsh , E. Elitsur , M. C. Vianna , D. S. Lindsay , and S. M. Reed
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EXPERIMENTAL INFECTION OF PONIES WITH *SARCOCYSTIS FAYERI* AND DIFFERENTIATION FROM *SARCOCYSTIS NEURONA* INFECTIONS IN HORSES

W. J. A. Saville, J. P. Dubey*, M. J. Oglesbee†, C. D. Sofaly, A. E. Marsh, E. Elitsur, M. C. Vianna*, D. S. Lindsay‡, and S. M. Reed§

Department of Veterinary Preventive Medicine, College of Veterinary Medicine, Ohio State University, Columbus, Ohio 43210-1092. *e-mail: saville.4@osu.edu*

ABSTRACT: *Sarcocystis neurona* and *Sarcocystis fayeri* infections are common in horses in the Americas. Their antemortem diagnosis is important because the former causes a neurological disorder in horses, whereas the latter is considered nonpathogenic. There is a concern that equine antibodies to *S. fayeri* might react with *S. neurona* antigens in diagnostic tests. In this study, 4 ponies without demonstrable serum antibodies to *S. neurona* by Western immunoblot were used. Three ponies were fed 1×10^5 to 1×10^7 sporocysts of *S. fayeri* obtained from dogs that were fed naturally infected horse muscles. All ponies remained asymptomatic until the termination of the experiment, day 79 postinoculation (PI). All serum samples collected were negative for antibodies to *S. neurona* using the Western blot at the initial screening, just before inoculation with *S. fayeri* (day 2) and weekly until day 79 PI. Cerebrospinal fluid samples from each pony were negative for *S. neurona* antibodies. Using the *S. neurona* agglutination test, antibodies to *S. neurona* were not detected in 1:25 dilution of sera from any samples, except that from pony no. 4 on day 28; this pony had received 1×10^7 sporocysts. Using indirect immunofluorescence antibody tests (IFATs), 7 serum samples were found to be positive for *S. neurona* antibodies from 1:25 to 1:400 dilutions. *Sarcocystis fayeri* sarcocysts were found in striated muscles of all inoculated ponies, with heaviest infections in the tongue. All sarcocysts examined histologically appeared to contain only microcytes. Ultrastructurally, *S. fayeri* sarcocysts could be differentiated from *S. neurona* sarcocysts by the microtubules (mt) in villar protrusions on sarcocyst walls; in *S. fayeri* the mt extended from the villar tips to the pellicle of zoites, whereas in *S. neurona* the mt were restricted to the middle of the cyst wall. Results indicate that horses with *S. fayeri* infections may be misdiagnosed as being *S. neurona* infected using IFAT, and further research is needed on the serologic diagnosis of *S. neurona* infections.

Horses in North America are infected with 2 *Sarcocystis* species, i.e., *Sarcocystis fayeri* and *Sarcocystis neurona*. *Sarcocystis fayeri* infections occur in approximately 30% of horses based on early research (Dubey et al., 1977), whereas approximately 50% of horses are exposed to *S. neurona* (Bentz et al., 1997; Blythe et al., 1997; Saville et al., 1997; Dubey, Lindsay, Saville et al., 2001).

Horses with *S. fayeri* infections may exhibit no clinical sign, or they may have mild to severe chronic illness with anemia, muscle soreness, and malnourishment (Tinling et al., 1980; Fayer and Dubey, 1982; Cawthorn et al., 1990). In contrast, equine protozoal myeloencephalitis, caused by *S. neurona* infections, is 1 of the most important and severe neurologic diseases affecting horses (Dubey, Lindsay, Saville et al., 2001). This becomes problematic because of the confusion in diagnosing either disease. Originally, a Western blot analysis was developed to detect antibodies to *S. neurona* in horses, which was reported to be able to differentiate between *S. neurona* and *S. fayeri* (Granstrom et al., 1993). Recently, another group of scientists reported that the Western blot analysis for *S. neurona* antibodies did not perform as well as an indirect immunofluorescence antibody test (IFAT) (Duarte et al., 2003).

The purpose of this study was to infect horses with *S. fayeri* sporocysts and follow them over time to demonstrate the ability to differentiate these infections from *S. neurona* infections.

MATERIALS AND METHODS

Collection of *Sarcocystis fayeri* sporocysts and preparation of horse inoculum

Muscles were collected from 20 horses from a slaughterhouse in Texas, and about 2 kg was fed to 3 laboratory-raised dogs. The dogs were killed 12 days later, and the sporocysts were collected from intestinal scrapings suspended in antibiotics, as described previously (Dubey et al., 1989). Sporocysts were counted using a hemacytometer and suspended in a concentration of 1×10^6 sporocysts/ml. Sporocyst inocula were shipped from Beltsville, Maryland, to the Ohio State University (OSU), Columbus, Ohio, for inoculation of the horses. Sporocysts had been stored for 163 days at 4 C before being fed to ponies.

Experimental infection of horses

Four pony or pony-crossed weanling foals between 4 and 5 mo of age were purchased from a local horse dealer after testing negative for antibodies to *S. neurona* using Western blot analysis (Granstrom et al., 1993). All 4 ponies were males that ranged in weight from 55 to 97 kg. The ponies were housed in the large-animal facility in the University Laboratory Animal Resource Center at the College of Veterinary Medicine, OSU. The control pony (no. 2) was housed in a separate stall. All ponies were fed heat-treated pellets (Buckeye Feeds, Dalton, Ohio) and autoclaved hay, as well as water, ad libitum.

All 4 ponies were randomly assigned to a treatment and subjected to complete physical examination on the day of arrival (day 0). Blood was collected from all ponies using vacutainer serum separator tubes. Each pony was sedated with xylazine (Sedazine®, Fort Dodge Animal Health, Fort Dodge, Iowa) and Torbugesic® (Fort Dodge Animal Health) for cerebrospinal fluid (CSF) collection by standing lumbosacral puncture as described previously (Green et al., 1992). Aliquots of serum and CSF were submitted to a commercial laboratory (Equine Biodiagnostics, Inc., Lexington, Kentucky) for Western blot analysis to detect antibodies to *S. neurona*. After CSF collection, ponies nos. 1, 3, and 4 were administered assigned 100,000, 1×10^6, and 1×10^7 *S. fayeri* sporocysts, respectively, by nasogastric intubation. Pony no. 2 was administered an equal volume of normal saline as a placebo.

The ponies were observed daily for general health (temperature, pulse, and respiratory rate) and appetite. Serum samples were collected weekly for detection of *S. neurona* antibodies. Just 24 hr before being

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* Animal Parasitic Diseases Laboratory, Animal and Natural Resources Institute, U.S. Department of Agriculture, Agricultural Research Service, Beltsville, Maryland 20705-2350.

† Department of Veterinary Biosciences, College of Veterinary Medicine, Ohio State University, Columbus, Ohio 43210-1092.

‡ Department of Biomedical Sciences and Pathobiology, Center for Molecular Medicine and Infectious Disease, Virginia–Maryland Regional College of Veterinary Medicine, Virginia Tech, 1410 Prices Fork Road, Blacksburg, Virginia 40061-1092.

§ Department of Large Animal Medicine, College of Veterinary Medicine, Ohio State University, Columbus, Ohio 43210-1092.

TABLE I. Detection of antibodies to *Sarcocystis neurona* in sera of ponies fed *S. fayeri* sporocysts.

Pony no.	Dose	Day PI	*S. neurona* antibodies		
			Immunoblot*	SAT†	IFAT‡
1	10^5	2	Negative	<25	<25
		37	Negative	<25	25
		79	Negative	<25	100
2	None	2	Negative	<25	<25
		37	Negative	<25	<25
		79	Negative	<25	25
3	10^6	2	Negative	<25	<25
		37	Negative	<25	400
		79	Negative	<25	100
4	10^7	2	Negative	<25	<25
		37	Negative	1:50†	100
		79	Negative	<25	100

* Western blot by Equine Biodiagnostics, Inc. (Granstrom et al., 1993); no reaction.
† *Sarcocystis neurona* agglutination test (Lindsay and Dubey, 2001).
‡ Indirect immunofluorescence antibody test (Duarte et al., 2003).

killed, serum and CSF samples of all ponies were collected. After death with Beuthanasia®-D (Schering-Plough, Union, New Jersey), a complete postmortem examination was performed on all ponies except the control pony. The control pony was donated to 1 of the authors.

Postmortem examination of experimental horses

During postmortem examination, samples of bladder, spleen, pancreas, kidney, liver, adrenal, heart, lung, diaphragm, quadriceps muscles, and tongue were fixed in 10% buffered neutral formalin and processed for routine examination of hematoxylin and eosin–stained sections after paraffin embedding. Retrospectively, formalin-fixed pieces of tongue from pony no. 4 were prepared for transmission electron microscopy (TEM) as described previously (Stanek et al., 2002).

Immunohistochemical examination

Paraffin-embedded sections of tongue from all 3 ponies fed *S. fayeri* were reacted with *S. neurona* antibodies in an immunohistochemical test (Stanek et al., 2002).

Serological examination for antibodies to *Sarcocystis neurona*

Antibodies to *S. neurona* were assessed in sera of ponies by Western immunoblot, *S. neurona* agglutination test (SAT), and IFAT. The immunoblots were performed at Equine Biodiagnostics, Inc., as described by Granstrom et al. (1993). SAT was performed at Virginia Tech, Blacksburg, Virginia, as described by Lindsay and Dubey (2001), and all 12 weekly samples from each pony were examined. Samples examined for SAT were initially screened at 1:25 dilution, and positive samples were titrated. IFAT was performed at the University of California Veterinary Teaching Hospital, Davis, California, as described by Duarte et al. (2003). For IFAT, 3 serum samples from each pony (days 2, 37, and 79 of the experiment) were examined using 1:25, 1:100, and 1:400 dilutions on each serum. SAT and IFAT were performed blindly without knowing the pony number or whether they were treated ponies or controls.

RESULTS

Experimental horse results

All serum samples collected were negative for antibodies to *S. neurona* using Western blot at initial screening, just before inoculation with *S. fayeri* (day 2) and weekly until day 79 postinoculation (PI). Both CSF samples collected on day 2 and day 82 PI from each pony were negative for *S. neurona* antibodies. Antibodies to *S. neurona* were not detected in 1:25 dilution of sera from any sample except in 1 case. The exception was the sample collected from pony no. 4 on day 28 that was positive at 1:50 dilution. Results of IFAT are given in Table I.

Sarcocysts were found in striated muscles of all inoculated ponies. Tongue contained the most numbers of sarcocysts (Fig. 1). In histologic sections, sarcocysts were up to 40 μm wide and up to 1,000 μm long. All sarcocysts examined histologically appeared to contain only metrocytes and no bradyzoites (Figs. 1, 2). The sarcocyst wall was up to 4 μm wide (Fig. 1A), and sarcocysts with cyst wall of different widths were found in the same section (Fig. 1A). By light microscopy, the cyst wall was found to be striated. Sarcocysts from all 3 ponies did not stain with *S. neurona* antibodies.

Ultrastructurally, the sarcocyst wall consisted of villar pro-

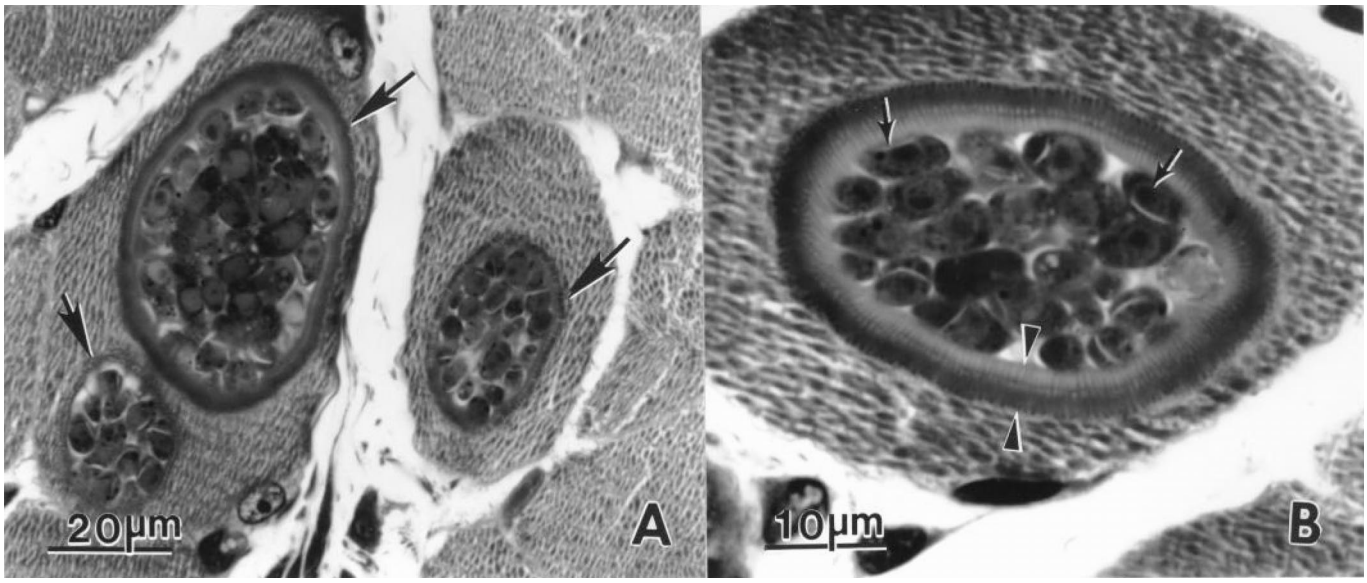

FIGURE 1. Sections of sarcocysts of *Sarcocystis fayeri* in tongue of pony no. 4. Toluidine blue stain. (**A**) Three sarcocysts with varying thickness of the cyst wall (arrows). (**B**) Sarcocyst with thick cyst wall (arrowheads) and metrocysts (arrows).

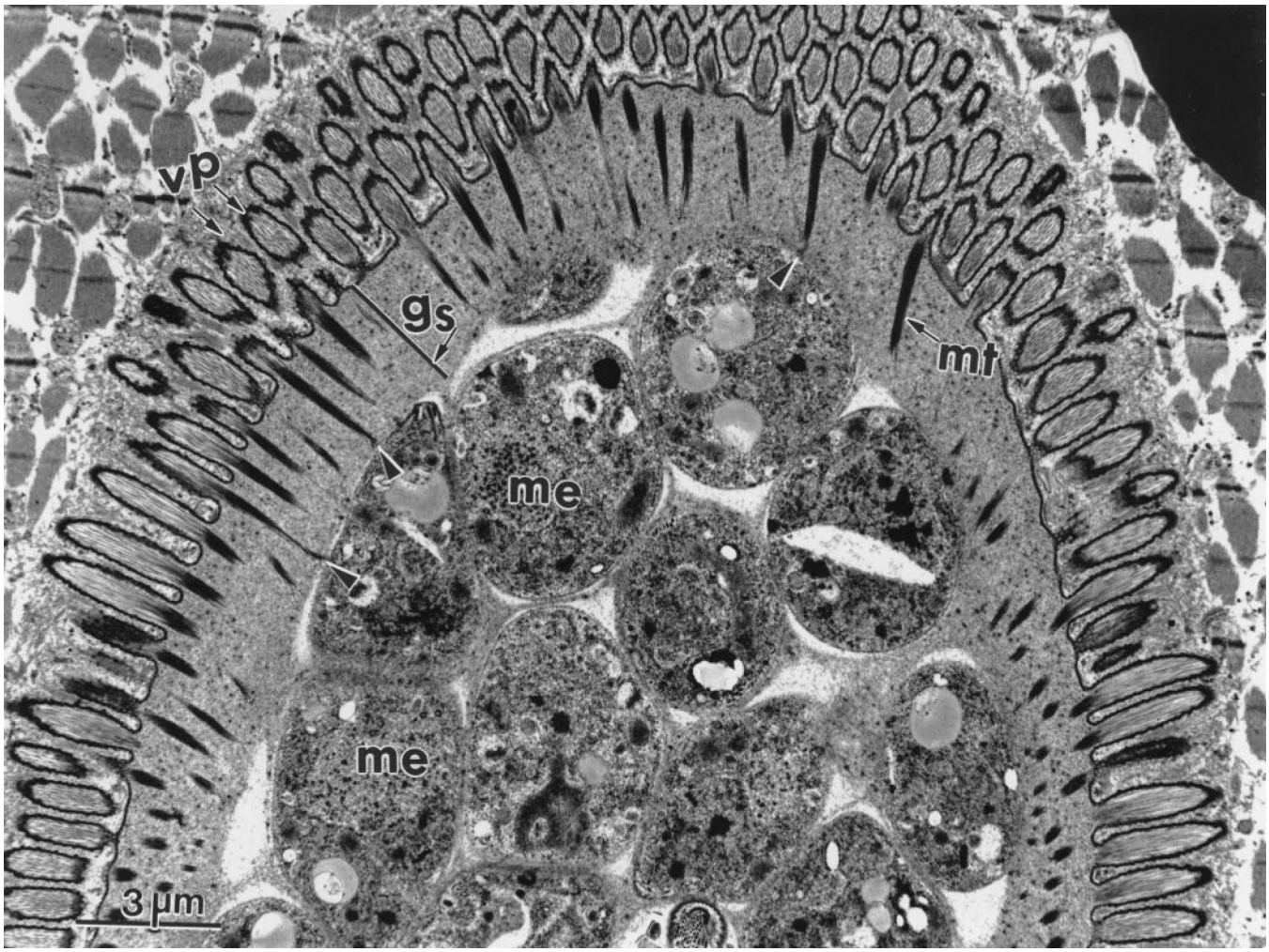

FIGURE 2. Transmission electron micrograph of a sarcocyst of *Sarcocystis fayeri*. Note elongated villar protrusions (vp), with microtubules (mt) that extend into the ground substance (gs) up to the pellicle of metrocytes (me) (arrowheads). Only me and no bradyzoite was seen in this cyst.

trusions (vp) and the ground substance (gs) layer. The parasitophorous vacuolar membrane (pvm), the outermost membrane covering vp, was embedded into the host cell cytoplasm (Figs. 2, 3). The pvm was lined by an electron-dense layer (edl), which varied in density and width. The edl was thickest and most dense at the tip than at the base of the vp (Fig. 3). Porelike structures were formed by the thinness of the edl at the base of the vp. The edl was up to 90 nm thick at the tip of the vp.

Each vp contained prominent microtubules (mt) that extended from the tip to the base and even up to the pellicle of metrocytes. The structure of the mt varied depending on the location in the sarcocyst. Mt in the vp had no visible electron-dense granule. The mt were unevenly spaced in the vp. Mt in the gs layer were tightly packed and converged toward the pellicle of the metrocytes. They were remarkably more electron dense in the gs layer than in the vp. The gs layer was up to 2.5 μm wide and relatively devoid of electron-dense structures, except for the presence of mt.

The metrocytes were approximately 8 × 5 μm in size. No bradyzoite was observed using TEM.

DISCUSSION

This study demonstrates that we are able to distinguish *S. fayeri* from *S. neurona* infections in horses after experimental infection with *S. fayeri* sporocysts. This can be accomplished using the Western blot analysis as described by Granstrom et al. (1993). There appears to be some cross-reactivity using both SAT and IFAT in some instances.

SAT was positive in only 1 instance, on day 37 PI, at 1:50 dilution; however, this horse received the highest dose (1×10^7) of sporocysts. On the other hand, IFAT was positive at 1:100 in all 3 inoculated ponies and 1:400 in 1 pony. Duarte et al. (2003) considered a titer of 1:80 as a specific IFAT titer for *S. neurona* based on data from naturally infected horses. Further research is needed to assess whether the severity of *S. fayeri* infection affects the cross-reactivity to *S. neurona*.

The ultrastructure of *S. fayeri* sarcocysts in this study is remarkably similar to that of *S. fayeri*–like sarcocysts in a naturally infected horse from California described in detail by Tinling et al. (1980), with 1 exception. Tinling et al. (1980) reported

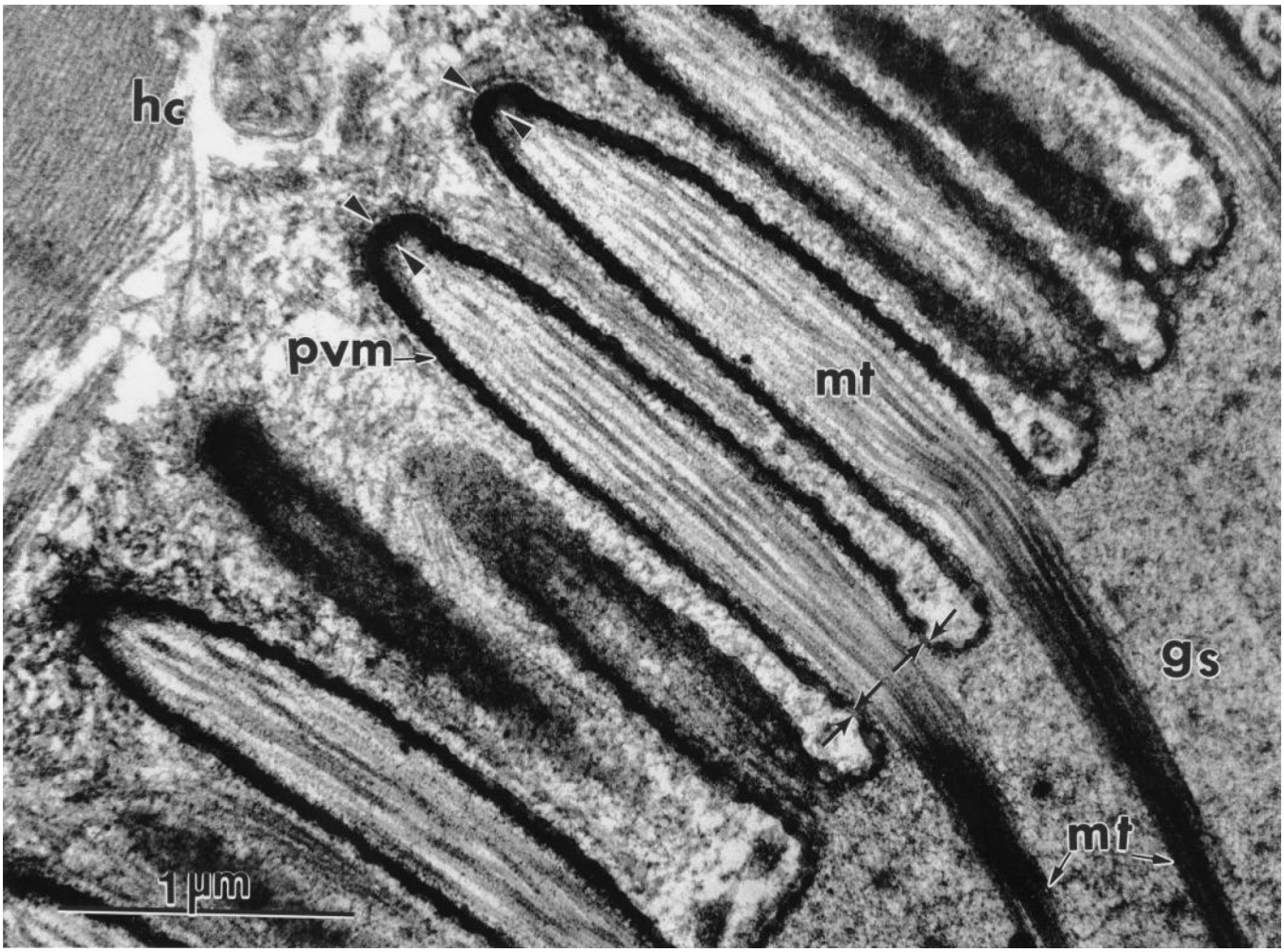

FIGURE 3. Transmission electron micrograph of a sarcocyst of *Sarcocystis fayeri*. Note details of the villar protrusions (vp) extending into the host cell (hc). The parasitophorous vacuolar membrane (pvm) is lined by an electron-dense layer that is thicker at the villar tips (arrowheads) and narrow at the base of the vp (arrowheads). Microtubules (mt) extend from the tip to the base of the vp into the ground substance (gs), and they are more electron dense in the gs than in the vp.

linearly arranged electron-dense structures in mt. These bodies were not seen in this study. Cawthorn et al. (1990) also found these electron-dense structures in another naturally infected horse from Canada. Specimens in this study were fixed in formalin, whereas Tinling et al. (1980) and Cawthorn et al. (1990) fixed specimens in glutaraldehyde. Whether these procedures used or the stage of the maturity of sarcocysts examined made the difference is unknown. Sarcocysts in this study were immature, whereas in the other 2 studies they were mature. It was surprising to find only immature sarcocysts in this study in ponies killed 79 days PI. In our previous study, sarcocysts had matured in a pony killed 77 days PI (Fayer and Dubey, 1982).

In general, the sarcocysts of *S. fayeri* appeared similar to those of *S. neurona* in tissues of cats and raccoons fed *S. neurona* sporocysts from the feces of opossums (Dubey, Lindsay, Fritz et al., 2001; Stanek et al., 2002). However, there were differences in vp of these parasites. The villar tips of *S. fayeri* were stubby, whereas those in *S. neurona* were more pointed. Mt in the gs layer were not as electron dense in *S. neurona* as in *S. fayeri*. Although most sarcocysts were immature, sarcocysts of *S. fayeri* did not react with antibodies to *S. neurona*. We are aware that staining of sarcocysts with antibodies raised against merozoites may vary. Immature sarcocysts of *S. neurona* reacted well with *S. neurona* antibodies, and this reactivity was diminished with the maturation of sarcocysts (Stanek et al., 2002). It is important to recognize these differences because *S. neurona* sarcocysts have not yet been documented in horses.

In conclusion, this study has demonstrated that infections with *S. fayeri* and *S. neurona* in horses can be distinguished using Western blot testing of sera for antibodies to *S. neurona* and that the sarcocysts of the 2 species are structurally different enough to distinguish them using microscopy. Because of its varied presentation and high seroprevalence, we are still faced with uncertainty in the diagnosis of clinical *S. neurona* infections. Consequently, much more research is necessary in this area to develop more specific testing modalities.

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