

Development of *Sarcocystis falcatula* in cell cultures demonstrates that it is different from *Sarcocystis neurona*

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SUMMARY

The development of *Sarcocystis falcatula* merozoites in bovine turbinate (BT) cell cultures is described and compared with development of *Sarcocystis neurona* merozoites. Merozoites of *S. falcatula* entered BT cell cultures and increased in size until 3 days post-inoculation when the nucleus of some merozoites developed lobes. Developing schizonts present at 4 days contained a lobed nucleus or appeared multinucleate. A single mature schizont was observed 4 days p.i. Schizonts were numerous 5 and 6 days p.i. Merozoites were produced from blastophores on the schizont. *S. neurona* merozoites developed to mature schizonts by 3 days p.i. in BT cells and a residual body was often present. Transmission electron microscopy revealed that *S. falcatula* merozoites possessed more micronemes than did *S. neurona* merozoites. Our study demonstrates that *S. falcatula* and *S. neurona* are not the same parasite.

Key words: *Sarcocystis falcatula*, *Sarcocystis neurona*, coccidia, schizonts, merozoites, cell culture.

INTRODUCTION

Sarcocystis falcatula Stiles, 1893 is a protozoan parasite with birds as intermediate hosts and the opossum, *Didelphis virginiana* as the definitive host (Box & Duszynski, 1978). It has an unusually wide intermediate host range for a *Sarcocystis* species (Box & Smith, 1982). *Sarcocystis falcatula* is highly pathogenic for several species of pet birds and outbreaks of fatal infections have been reported in birds raised in outdoor aviaries (Clubb & Frenkel, 1992) and in collections of psittacines in zoos (Hillyer *et al.* 1991). The opossum is its only known definitive host for *S. falcatula* (Box, Meier & Smith, 1984).

Equine protozoal myeloencephalitis (EPM) is a neuralgic syndrome seen in horses from the Americas and is caused by infection with *S. neurona* Dubey, Davis, Speer, Bowman, de Lahunta, Granstrom, Topper, Hamir & Suter, 1991 (Dubey *et al.* 1991). The condition has been recognized for over 20 years and EPM is the most important protozoal disease of horses in the United States (MacKay, 1997). Recent

serological surveys indicate that 45–53% of horses have antibodies to *S. neurona* (Bentz, Gramstrom & Stamper, 1997; Blythe *et al.* 1997; Saville *et al.* 1997).

Studies using the small subunit ribosomal RNA sequences have indicated that *S. falcatula* and *S. neurona* were the same parasite (Dame *et al.* 1995; Fenger *et al.* 1995). However, conclusive biological demonstration that the two are the same species is lacking. Results of the study reported here do not support the assumption that the two species are the same. We demonstrate that the *in vitro* development of *S. falcatula* is different from that of *S. neurona*.

MATERIALS AND METHODS

Sarcocystis neurona SN2 strain merozoites (Davis, Daft & Dubey (1991) isolated from a horse with EPM and *S. falcatula* Cornell strain merozoites (isolated from the lungs of a budgerigar, *Melopsittacus undulatus*, fed sporocysts from an opossum) were grown and maintained in bovine turbinate cells (BT cells, ATTC CRL 1390, American Type Culture Collection, Rockville, Maryland, USA). The BT cells were grown to confluence in 25 cm² plastic cell culture flasks in growth medium consisting of 10% (v/v) foetal bovine serum (FBS) in RPMI-1640 medium supplemented with 100 U penicillin G/ml, and 100 mg streptomycin/ml. Cell

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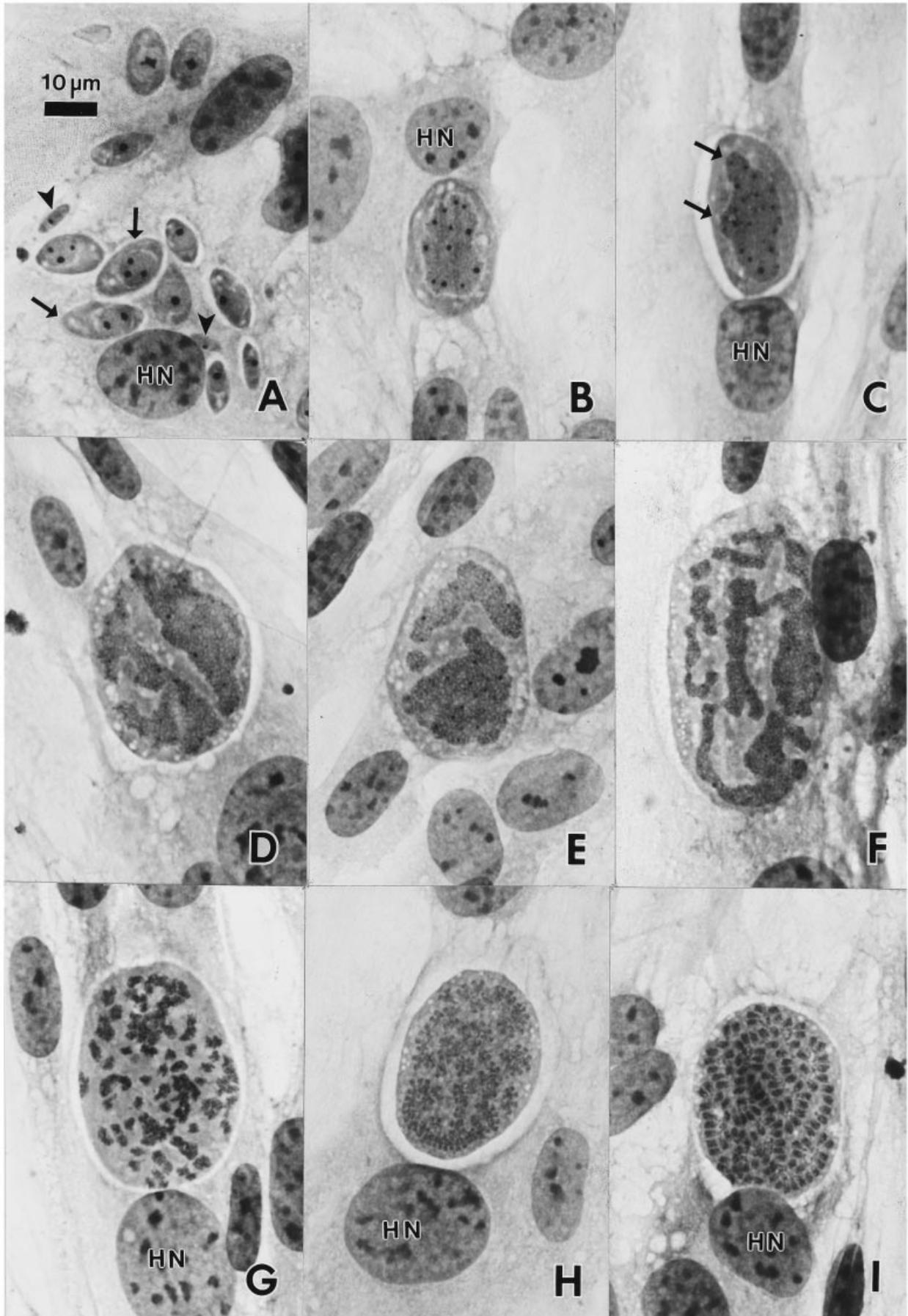


Fig. 1. For legend see opposite.

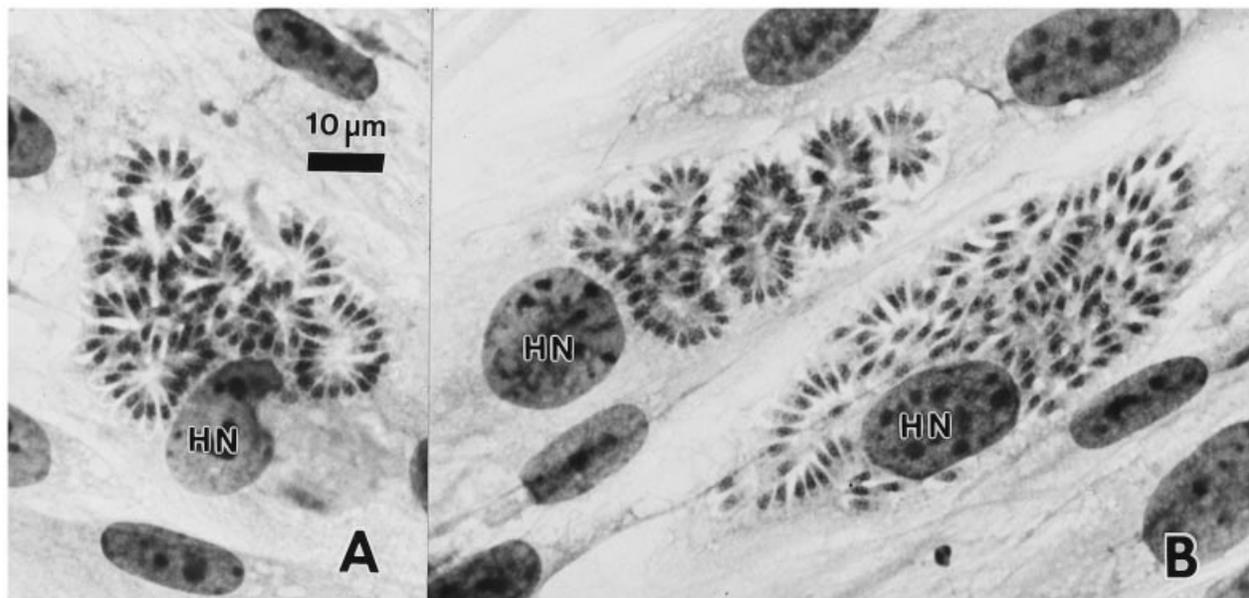


Fig. 2. (A and B) Mature schizonts of *Sarcocystis falcatula*. All 6 days p.i. (A) Schizont demonstrating merozoites originating from 4-5 blastophores. Note the absence of a residual body. (B) Schizont demonstrating merozoites originating from 8 blastophores directly above a schizont that contains merozoites which no longer appear to be associated with blastophores. HN, host cell nucleus.

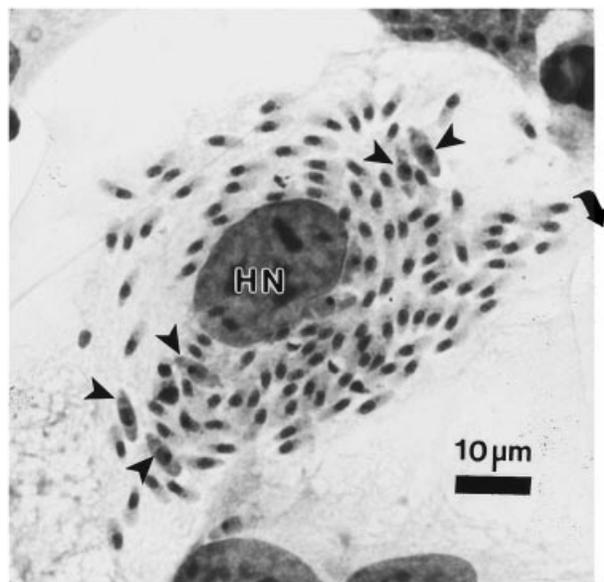


Fig. 3. Mature schizont of *Sarcocystis falcatula* in which the merozoites are arranged in a circular pattern around the host cell nucleus (HCN). Several of the merozoites (arrowheads) have begun to enlarge. Six days p.i. HN, host cell nucleus.

cultures were maintained in growth medium in which the FBS content was lowered to 2%. Cell cultures were incubated at 37 °C in a humidified atmosphere containing 5% CO₂ and 95% air. For descriptive studies, merozoites were harvested from infected cell cultures by removing the medium and replacing it with Hanks' balanced salt solution without calcium and magnesium. The BT cells were then removed from the plastic growth surface by use of a cell scraper. This cell mixture was passed through a 27-gauge needle attached to a 10 ml syringe to rupture host cells. The suspension was then filtered through a sterile 3 µm filter to remove cellular debris. The number of merozoites in the filtrate was determined using a haemocytometer. From 0.2 to 1.0 × 10⁶ merozoites of *S. falcatula* were used as inoculum and from 1.0 to 2.0 × 10⁵ merozoites of *S. neurona* were used. The merozoites were inoculated onto 22 mm² glass coverslips containing a monolayer of BT cells in 6-well tissue culture plates.

Cover-slips were removed on days 1, 2, 3, 4, 5 and 6 p.i., fixed in 10% phosphate-buffered formalin for 30 min, placed in 100% methanol for 10 min and stained with a Giemsa-type stain. Cover-slips were

Fig. 1. (A-I) Development from merozoites to immature schizonts of *Sarcocystis falcatula*. All 6 days p.i. (A) Multiply infected cell that demonstrates merozoites (arrowheads) and several developing schizonts. Note the 2-3 prominent nucleoli in some of the larger stages (arrows). (B) Developing schizont in which the nucleus occupies about 80% of the cytoplasm and contains 12 nucleoli. (C) Schizont with a nucleus that is beginning to develop lobes (arrows). (D and E) Schizonts, each with a nucleus that is highly folded, giving the appearance of grooves in the nucleus. (F) Advanced schizont demonstrating more pronounced nuclear changes than shown in (D) or (E). (G and H) Schizonts each with a nucleus that is in advanced stages of condensing. (I) Schizont with numerous nuclei that have segregated into developing merozoites. HN, host cell nucleus.

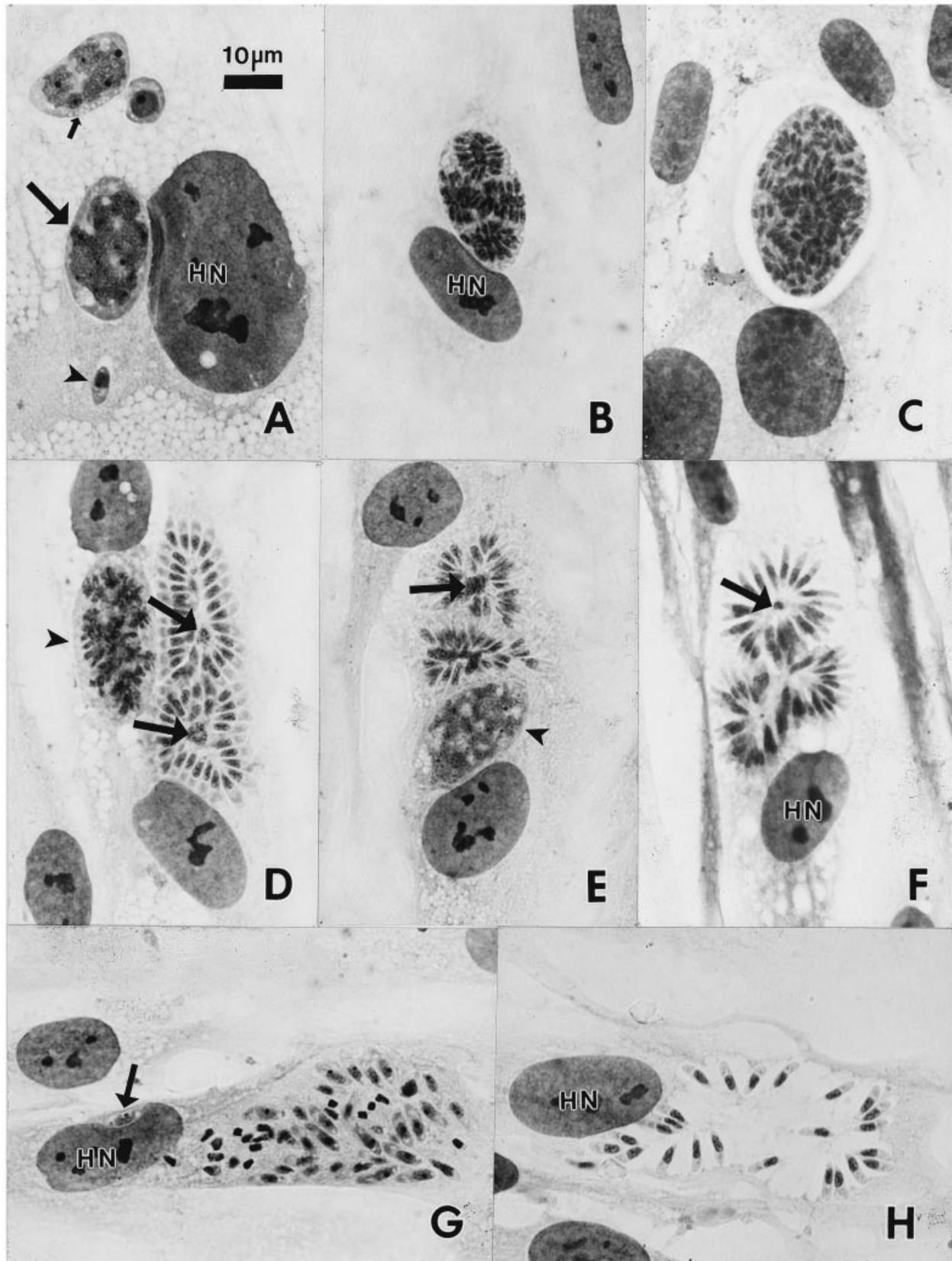


Fig. 4. (A–H) Developing and mature schizonts of *Sarcocystis neurona*. All 6 days p.i. (A) Host cell containing a merozoite (arrowhead), a developing schizont (small arrow) and a developing schizont with a lobed nucleus (large arrow). (B and C) Developing schizonts demonstrating condensing nuclei prior to merozoite formation. (D and E) Developing schizonts (arrowheads) adjacent to mature schizonts with merozoites being produced at blastophores. Note the residual body (arrows). (F) Mature schizont clearly demonstrating a residual body (arrow). (G) Host cell that contains randomly arranged merozoites. Note a merozoite (arrow) has indented the host cell nucleus. (H) Host cell similar to (G) in which a majority of the merozoites has escaped. HN, host cell nucleus.



Fig. 5. (A and B) TEM of merozoites of *Sarcocystis falcatula* and *Sarcocystis neurona*. (A) Longitudinal section through *S. falcatula*. Note the conoid (arrowhead), absence of rhoptries, and the numerous micronemes which extend three-quarters of the way to the nucleus (arrow). (B) Longitudinal section through *S. neurona*. Note the conoid (arrowhead), absence of rhoptries, and the numerous micronemes which extend one-quarter of the way to the nucleus (arrow).

attached to glass microscope slides with Permount® (Fisher Scientific, Fairlawn, New Jersey, USA) and examined with light microscopy. Stages were measured with a calibrated ocular micrometer. Measurements are expressed in μm as the mean followed by the range and number (N) of stages measured in parenthesis.

Transmission electron microscopy was done on cultures 16 days p.i. The BT cells were scraped from the growth surface, pelleted by centrifugation, and fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2). The pellet was post-fixed in 2% (w/v) osmium tetroxide, dehydrated in a series of ethanols, passed twice through propylene oxide and embedded in Epon/Araldite (Poly-science, Inc., Warrington, Pennsylvania, USA). Thin sections were stained in uranyl acetate and lead citrate and examined using a Philips TEM 201 transmission electron microscope operating at 80 kV.

RESULTS

Sarcocystis falcatula merozoites entered BT cells and underwent schizogony within 4 days (Figs 1–3).

Parasites were usually located next to the host cell nucleus. Occasionally parasites appeared to be in the host cell nucleus. Division was asynchronous resulting in cells containing structurally different organisms. Intracellular merozoites measuring 7.9 by 2.4 (6.4–10.4 by 1.6–4.0, $N = 20$) were the only stages observed for *S. falcatula* 1 day p.i. Merozoites grew in size and were 10.6 by 5.1 (8.0–16.0 by 1.6–8.0, $N = 20$) 2 days p.i. The nucleus of some merozoites became lobed (Fig. 1) by 3 days p.i. and these developing schizonts were 17.4 by 10.4 (10.4–30.4 by 4.6–18.4, $N = 25$). The nucleoli were prominent in these stages. The nucleus of schizonts underwent additional transformations to form groves, lobes and projections and eventually portions of the nucleus were incorporated into merozoites as a single nucleus. A single mature schizont was seen 4 days p.i. Four merozoites were 5.6 by 1.6. They contained a posteriorly located nucleus. Numerous developing schizonts and mature schizonts were seen 5 and 6 days p.i. (Figs 2 and 3). Merozoites were formed at blastophores which developed on the schizont surface prior to cytokinesis. A residual body was not seen. Developing schizonts present 5 days p.i. were

37.3 by 24.6 (33.6–44.0 by 20.0–30.4, $N = 20$). Merozoites present 5 days p.i. were 6.3 by 1.6 (5.6–7.2 by 1.2–2.4, $N = 20$). Developing schizonts present 6 days p.i. were 36.6 by 24.6 (27.2–44.0 by 17.6–32.0, $N = 20$). Merozoites present 6 days p.i. were 6.6 by 1.8 (5.6–8.0 by 1.6–2.4, $N = 20$).

Sarcocystis neurona merozoites entered BT cells and underwent schizogony within 3 days (Fig. 4). Parasites were usually located next to the host cell nucleus. Occasionally parasites appeared to be in the host cell nucleus. Division was asynchronous resulting in cells containing structurally different organisms. Intracellular merozoites measuring 7.9 by 2.9 (6.4–11.2 by 1.6–4.0, $N = 20$) were the only stages observed for *S. neurona* 1 day p.i. Merozoites increased in size and became developing schizonts. Merozoites with a lobed nucleus were observed 2 days p.i. and these developing schizonts were 12.9 by 5.4 (8.8–20.8 by 2.4–11.2, $N = 20$). Developing and mature schizonts were present at 3 days p.i. The developing schizonts were 24.6 by 12.9 (16.0–32.8 by 7.2–17.6, $N = 20$). Merozoites in mature schizonts had a posteriorly located nucleus and were 6.7 by 1.5 (5.6–7.2 by 1.2–1.6, $N = 15$) at 3 days p.i. Merozoites were formed at blastophores which developed on the schizont surface prior to cytokinesis. A residual body was often visible in mature schizonts observed 3–6 days p.i. Developing schizonts present at 4 days were 27.6 by 15.2 (20.0–41.6 by 11.2–18.4, $N = 20$). Merozoites in mature schizonts were 5.8 by 1.7 (4.8–6.4 by 1.6–2.4, $N = 20$) at 4 days p.i. Developing schizonts present at 5 days were 26.2 by 14.2 (20.0–40.0 by 11.2–17.6, $N = 20$). Merozoites in mature schizonts were 5.6 by 1.7 (4.8–6.4 by 1.6–2.4, $N = 20$) at 5 days p.i. Developing schizonts present at 6 days were 24.2 by 13.7 (17.6–30.4 by 10.4–18.4, $N = 20$). Merozoites in mature schizonts were 5.8 by 1.6 (4.8–6.4 by 1.6, $N = 20$) at 6 days p.i.

Transmission electron microscopy revealed that both parasites developed in the cytoplasm of the host cell. Merozoites of *S. falcatula* (Fig. 5) contained a conoid, lacked rhoptries, had numerous micronemes which extended three-quarters of the way to the nucleus and had 22 subpellicular microtubules and other organelles typical for apicomplexan merozoites. Merozoites of *S. neurona* (Fig. 5) contained a conoid, lacked rhoptries, had micronemes which extended one-quarter the way to nucleus and had 22 subpellicular microtubules and other organelles typical for apicomplexan merozoites.

DISCUSSION

Our study demonstrates structural and developmental differences between *S. falcatula* and *S. neurona* using light microscopy and TEM. The most striking difference observed with light microscopy was the larger size of developing *S. falcatula* schizonts, the longer developmental period needed

for schizogony of *S. falcatula*, and the absence of a residual body in mature *S. falcatula* schizonts. Our TEM observations indicate that *S. falcatula* has more micronemes and they extend farther towards the merozoite nucleus than the micronemes of *S. neurona*. Results of our study do not support the contention based on small subunit ribosomal RNA sequences (Dame *et al.* 1995; Fenger *et al.* 1995) that the 2 parasites are the same. Additional genetic analysis of *S. falcatula* and *S. neurona* is needed to determine if genetic markers can be used to differentiate the 2 species.

Research on potential vaccines for EPM should be done with isolates that come from clinically ill horses. This will assure that the appropriate immunodominant antigens will be identified. Likewise, generation of cDNA or gDNA libraries for potential vaccines should be done with isolates of *S. neurona* from clinically ill horses.

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