ISOLATES OF SARCOCYSTIS FALCATULA–LIKE ORGANISMS FROM SOUTH AMERICAN OPOSSUMS DIDELPHIS MARSUPIALIS AND DIDELPHIS ALBIVENTRIS FROM SÃO PAULO, BRAZIL


Published By: American Society of Parasitologists


URL: http://www.bioone.org/doi/full/10.1645/0022-3395%282001%291449%3AIOSFLO%5D2.0.CO%3B2
ISOLATES OF SARCOCYSTIS FALCATULA–LIKE ORGANISMS FROM SOUTH AMERICAN OPOSSUMS DIDELPHIS MARSPALIUS AND DIDELPHIS ALBIVENTRIS FROM SÃO PAULO, BRAZIL


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ABSTRACT: Isolates of Sarcocystis falcata–like organisms from South American opossums were characterized based on biological and morphological criteria. Sporocysts from intestinal scrapings of 1 Didelphis marsupialis and 8 Didelphis albiventeris from São Paulo, Brazil, were fed to captive budgerigars (Melopsittacus undulatus). Budgerigars fed sporocysts from all 9 isolates became ill and S. falcata–like schizonts were identified in sections of their lungs by immunohistochemical staining. Sarcocystis falcata–like organisms were cultured from lungs of budgerigars fed sporocysts from D. marsupialis and from lungs of budgerigars fed sporocysts from 3 of 8 D. albiventeris. The 33/54 locus amplified by polymerase chain reaction from culture-derived merozoites contained both a HinI endonuclease recognition site previously suggested to diagnose S. falcata and a DraI site though diagnosed S. neurona. Development of the isolate from D. marsupialis was studied in cell culture; its schizonts divided by endopolygeny, leaving a residual body. Morphological and genetic variation differentiated this Sarcocystis isolate originating in D. marsupialis from the Cornell 1 isolate of S. falcata. This is the first report of a S. falcata infection in the South American opossum, D. marsupialis.

Sarcocystis falcata (Stiles, 1893) causes severe disease to certain avian species. Several outbreaks of acute pulmonary sarcocystosis have been observed among species of Psittaciformes and Passeriformes from zoos in North America (Jacobson et al., 1984; Clubb et al., 1986; Smith, Craig et al., 1990; Hillyer et al., 1991; Clubb and Frenkel, 1992; Mirande et al., 1992; Page et al., 1992; Dubey, Garner et al., 2001). Experimentally, feeding even a few sporocysts can be lethal to certain avian species (Box and Smith, 1982; Dubey, 2000). Sarcocystis falcata is considered an unusual species of Sarcocystis because of its wide intermediate host range (Passeriformes, Psittaciformes, Columbiformes) and its prolonged schizogony (lasting up to 5 mo) (Smith et al., 1987a, 1987b; Neill et al., 1989; Smith, Neill et al., 1990).

The North American opossum Didelphis virginiana serves as a definitive host (Box and Duszynski, 1978; Duszynski and Box, 1978; Box and Smith, 1982; Box et al., 1984). Until 1995, S. falcata was considered the sole species to use D. virginiana as its definitive host. Recently, however, D. virginiana was found to be a definitive host for 2 additional nominal species of Sarcocystis: S. neurona (Dubey and Lindsay, 1998) and S. speeri (Dubey and Lindsay, 1999). Differentiation of these species was based on differences identified in their life cycles.

A genetic locus, termed 33/54, has been proposed to encompass variation enabling the differential diagnosis of S. neurona and S. falcata by means of polymerase chain reaction (PCR) amplification and restriction endonuclease digestion (Tanhauser et al., 1999). The PCR products from several isolates of S. neurona were cut into 884- and 216-bp fragments by DraI, whereas other isolates presumed to represent S. falcata were not; conversely, HinI failed to cut the products of S. neurona, but did cut those from S. falcata into 745- and 355-bp fragments (Tanhauser et al., 1999). Using this system to characterize 9 isolates of sporocysts from D. virginiana feces, 5 corresponded to the S. neurona pattern, 2 corresponded to the S. falcata pattern, but 2 were found to be cut by each of the 2 restriction endonucleases (Tanhauser et al., 1999).

Although these S. falcata–like parasites have begun to be characterized in D. virginiana, the single opossum species endemic to the continental United States, less is known about the diversity and distribution of such parasites among the several species of opossum endemic to Central and South America. Sarcocystis falcata–like parasites have been described in the South American opossum Didelphis albiventeris from Argentina and from Brazil (Dubey, Venturini et al., 1999; Dubey, Lindsay, Venturini, and Venturini, 2000; Dubey, Lindsay, Rezende, and Costa, 2000). The S. falcata–like parasites from Argentina, when assayed at the 33/54 locus, were found to contain both the DraI site thought to characterize S. neurona, as well as the HinI site thought to diagnose S. falcata. In contrast, the Brazilian isolate contained solely the DraI site (Dubey, Lindsay, Rezende, and Costa, 2000). To further evaluate the faunal diversity for Sarcocystis spp. among South American Didelphidae, we studied the morphological and molecular attributes of parasites obtained as sporocysts from 1 Didelphis marsupialis and 8 D. albiventeris, whose capacity to infect birds and failure to infect mice suggested their identity was S. falcata.

MATERIALS AND METHODS

Parasite sources

As part of a project to characterize isolates of S. falcata–like organisms in opossums, Sarcocystis sporocysts were obtained from scrapings of 1 D. marsupialis and 8 D. albiventeris intestine, as described by Dubey, Kerber et al. (2000) and Dubey, Lindsay, Kerber et al. (2001). Sporocysts from all opossums were initially fed to budgerigars (Melosittacus undulatus) and gamma interferon gene knockout (KO) mice to distinguish S. falcata from S. neurona and S. speeri. S. falcata is lethal to budgerigars but is not infective to KO mice (Marsh et al., 1997; Dubey and Lindsay, 1998), and S. neurona and S. speeri are lethal to KO mice but not infective to budgerigars (Dubey and Lindsay, 1998, 1999). Details of infections attributed to S. speeri and S. neurona from
these 9 opossums were reported earlier (Dubey, Kerber et al., 2000; Dubey, Lindsay, Kerber et al., 2001). The present paper discusses exclusively those parasites diagnosed as *S. falcata*–like on the basis of this bioassay.

**Sporocysts and bioassay**

*Sarcocystis* sp. sporocysts were obtained from 9 adult opossums submitted to the Universidade de São Paulo, Brazil, in November and December 1999 (Table I). These opossums inhabited the vicinity of São Paulo, Brazil, to Beltsville, Maryland. Sporocysts were stored at 4°C until used in the present studies. At Beltsville, sporocysts were bioassayed from infected cell cultures by removing the medium and replacing it with HBSS without calcium and magnesium. The BT cells were then removed from the plastic 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 numbers of merozoites in the filtrate were determined using a hemacytometer. The merozoites were inoculated onto 22-mm² glass coverslips containing a monolayer of CV-1 or BT cells in well-tissue culture plates. Coverslips were removed on 1–8, 10, and 11 days postinoculation (PI), fixed in 10% phosphate-buffered formalin for 30 min, placed in 100% methanol for 10 min, and stained with a Giemsa type stain. Coverslips were attached to glass microscope slides with Permount (Fisher Scientific, Fair Lawn, New Jersey) and examined with light microscopy. Developmental stages (*n* = 20) in CV-1 cells were measured with a calibrated ocular micrometer. Measurements are expressed as the mean followed by the range in parenthesis.

**Molecular characterization**

To preliminarily characterize the organisms newly isolated from *D. albiventris*, they were compared to other isolates of *Sarcocystis* spp. by means of a PCR amplification and restriction endonuclease digestion assay previously described by Tanhauser et al. (1999). DNA was extracted from merozoites that had been frozen at −20°C after harvest from infected CV-1 cells and subjected to PCR amplification using primers JNB33 (5'-CGAACAGAGATGAGGAAAAT-3') and JNB54 (5'-GGTTGTTGTGTGCTGATGTC-3') initially described by Tanhauser et al. (1999). These amplification products were subsequently subjected to restriction endonuclease *DraI* and *HinfI* run on a 1% agarose gel. These were compared to other parasite isolates resembling *S. falciata*; the SF-1A from budgerigar no. 62 of Dubey, Lindsay, Venturini, and Venturini (2000), an organism isolated from Jaboticabal, Brazil (bird no. 100 isolate described in Dubey, Lindsay, Rezende, and Costa, 2000; Dubey, Rosenthal, and Speer, 2001), and the Cornell 1 strain of *S. falcata* (Lindsay et al., 1999; Marsh et al., 1999). Two of these isolates (SF-1A, Cornell 1) were cloned by limiting dilution as described by Rosenthal et al. (2001). Two budgerigars (nos. 213, 217) were inoculated subcutaneously with ~10⁷ merozoites of the isolate of *S. falcata* recovered in culture from the lungs of budgerigar no. 200 (derived from *D. marsupialis*). Both budgerigars died of pulmonary sarcocystosis 9 days later. Lung homogenates from budgerigar no. 213 were used for sequencing studies. For comparative purposes, an *S. neurona* isolate (SN6 of Dubey, Matson et al., 1999) that had been cloned by limiting dilution was also assayed in this way. The large subunit (LSU) rDNA, the ITS-1 locus, and a locus of unknown location 25/396 were amplified by PCR from the *D. marsupialis*
isolate, directly sequenced by previously described means, and compared to all available homologs (Marsh et al., 1999; Tanhauser et al., 1999; Rosenthal et al., 2001).

RESULTS

Budgerigars fed sporocysts from all 9 opossums became ill and were killed or died by day 19 PI. Sarcocystis falcatula-like schizonts were found in lungs of all 26 budgerigars; these schizonts reacted positively with anti-S. falcatula polyclonal rabbit serum. Two control budgerigars remained clinically normal and protozoa were not detected in their tissues when examined 28 days PI.

Schizonts and merozoites were cultured from lungs of budgerigars fed sporocysts from each of the 9 opossums; these organisms resembled S. falcatula, but their development was not studied in detail. No structural differences were noted in developmental stages. The morphological development of organisms cultured from the lung of budgerigar no. 200, fed sporocysts from D. marsupialis no. 14, is considered representative. Merozoites entered CV-1 and BT cells and underwent schizogony within 3 days (Fig. 1). Merozoites were usually located next to the host cell nucleus. Intracellular merozoites and developing schizonts were seen on days 1 and 2 PI (Fig. 1A). Development and schizont nuclear division was asynchronous, resulting in cells containing structurally different organisms. Schizonts retained their elongate merozoite shape during development. Schizonts with mature merozoites were seen on days postinoculation but were more numerous 4 days PI. Merozoites formed peripherally and often were around a residual body (Fig. 1B). Merozoites present at 4 days PI measured 5.7 by 1.6 μm (4.8–7.2 by 1.2–1.6 μm, n = 20). Maturing schizonts with condensing nuclei measured 26.0 by 13.6 μm (22.4–29.6 by 11.2–16.0 μm, n = 20) 4 days PI. No additional developmental types were noted on the remaining observation days postinoculation.

Enough merozoites were available from cultures derived from 4 opossums (nos. 14, 15, 16, 20) to perform PCR amplification of the 33/54 locus (Fig. 2). The pattern of products produced by restriction digestion with DraI and HinfI of the parasites isolated from D. marsupialis were representative and were identical to that of SF-1A isolate (bird no. 62) in D. albiventris from Argentina and distinct from the SN6 isolate of S. neurona, the cloned Cornell 1 strain of S. falcatula, and bird no. 100 from Jabotical, Brazil.

The parasites derived from D. marsupialis were identical in sequence to the Cornell 1 strain of S. falcatula (Lindsay et al., 1999) and the SF-1A Argentine isolate (bird no. 62) in D. albiventris (Dubey, Lindsay, Venturini, and Venturini, 2000) at the 25/396 locus. In contrast, the ITS-1 sequenced from this D. marsupialis isolate contained unique substitutions but shared other substitutions heretofore found elsewhere only in UCD01 and the Cornell 2 isolates, but not in the Cornell 1 or Florida...
1 isolates of *S. falcataula* or in any of several exemplars of *S. neurona* described previously (Marsh et al., 1999). A single substitution differentiates this isolate in *D. marsupialis* from the Cornell 1 strain of *S. falcatula* over a span of the LSU rRNA gene that also differentiates *S. neurona* by a single substitution. To provide some context, several substitutions uniquely distinguish *S. speeri* at this highly conserved locus when it is compared to homologous sequences in *S. falcatula* and *S. neurona*. The ITS-1, 25/396, and LSU rRNA sequences have been deposited in GenBank (AF399339, AF389340, and AF389341, respectively).

**DISCUSSION**

Although accurate identification of parasites belonging to this group is hampered by a paucity of recognized genetic variation and the imperfect correspondence of variation in each locus, it can be concluded that *D. marsupialis* serve as a definitive host for parasites that are capable of infecting experimental avian hosts and that can also be distinguished from *S. neurona* and *S. speeri* on the basis of morphological and molecular characteristics. Their close relationship to, but evident distinction from, previously reported isolates identified as *S. falcatula* provides further indication that this taxon is diverse and perhaps consists of more than 1 species (Marsh et al., 1999; Tanhauser et al., 1999; Rosenthal et al., 2001).

Neither restriction endonuclease digestion of the 33/54 locus nor sequencing of the 25/396 locus distinguished the *S. falcataula*-like isolate (from *D. marsupialis* from São Paulo, Brazil) from previously reported isolates of *S. falcataula*-like isolates (from *D. albiventeris* from Argentina; SF-1A and SF-2A of Dubey, Lindsay, Venturini, and Venturini, 2000). At least 3 isolates from *D. albiventeris* from São Paulo, Brazil (from opossum nos. 15, 16, and 20 of this study), appear similar to the 2 isolates from *D. albiventeris* from Argentina on the basis of restriction endonuclease digestion of the 33/54 locus. Nonetheless, *D. marsupialis* and *D. albiventeris*-derived South American isolates differ by a small number of substitutions in the ITS-1 locus and by a single change in the LSU rDNA.

Budgerigars often die before sarcocysts are produced in experimental infections, and even those birds that survive infection may not develop mature sarcocysts (Box et al., 1984). Consequently, little data are available on the extent or nature of morphological variation among tissue stages of *S. falcataula*-like parasites. This limitation underscores the need for reliable molecular diagnostic tools but also hampers progress toward that goal. A full accounting of the diversity of this parasite fauna and the relationships among its members may require identification of experimental hosts for whom disease is more limited.

Both *D. albiventeris* and *D. marsupialis* are widely distributed in South and Central America, yet clinical pulmonary sarcocystosis among avian species has not been reported from this region. Results of the present and previous studies indicate, however, that *S. falcataula*-like parasites are prevalent. Natural avian infections might escape notice if they are generally asymptomatic. Alternatively, rapid scavenging of dead avian hosts may render natural mortality difficult to identify. Prevalent infection among widespread opossum species nonetheless underscores the risk to which birds, especially those in zoos, may be exposed.

**LITERATURE CITED**


DUBEY ET AL.—SARCOCYSTIS FALCATULA-LIKE ISOLATES FROM DIDELPHIS MARSUPIALIS AND D. ALBIVENTRIS 1453


