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Author(s): Angela E. Ellis, D.V.M., Ph.D., Elizabeth Mackey, D.V.M., Philip A. Moore, D.V.M., Ph.D., Dipl. A.C.V.O., Stephen J. Divers, B. VetMed, D. ZooMed, Dipl. A.C.Z.M., Dipl. E.C.Z.M., Patrick Hensel, D.M.V., Dipl. A.C.V.D., K. Paige Carmichael, D.V.M., Ph.D., Dipl. A.C.V.P., Peter Accola, D.V.M., Justin Brown, D.V.M., Ph.D., Nicole Gottdenker, D.V.M., Ph.D., Dip. A.C.V.P., M. Kevin Keel, D.V.M., Ph.D., Dipl. A.C.V.P., Barbara C. Shock, M.S., and Michael J. Yabsley, Ph.D.

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Abstract: *Besnoitia* spp. are coccidian parasites that infect a variety of wild and domestic mammals as well as some reptiles. Although infection with *Besnoitia* is common in Virginia opossums (*Didelphis virginiana*), clinical signs or death due to *Besnoitia* is rare. This manuscript describes four Virginia opossums that had severe clinical disease and inflammation associated with besnoitiosis. Clinical signs included trembling, incoordination, circling, blindness, poor body condition, and sudden death. Gross lesions included parasitic cysts in eyes, skin, and visceral organs. Histologically, cysts were often degenerate and associated with mild to marked inflammation, and amyloidosis was noted in one animal. Polymerase chain reaction and sequencing confirmed *Besnoitia darlingi* in three of the four opossums.

Key words: *Besnoitia*, besnoitiosis, coccidian, opossum, polymerase chain reaction (PCR).

INTRODUCTION

Besnoitia are coccidian parasites that infect a variety of wild and domestic mammals, as well as some reptiles.[1,2,5,6,9,12,14,17–19,22,24–28,34,35] At least two species, *Besnoitia wallacei* and *Besnoitia darlingi*, have been shown to have a heteroxenous life cycle in which domestic cats are the definitive hosts and rodents, lizards, or opossums serve as intermediate hosts.[32] The pathogenicity of *Besnoitia* is variable, and the underlying causes for this are poorly understood; however, young age, immunological naivete, immunosuppression, and stress have been suggested as predisposing factors for clinical disease.[11,15,22] Infection frequently causes no adverse effects, although animals may have numerous cysts in skin, striated muscle, and visceral organs. Histologic evidence of inflammation or clinical signs of disease have been reported in cattle, goats, caribou, reindeer, mule deer, miniature donkeys, Mexican burros, and a rabbit due to infection with various *Besnoitia* spp.[1,8,9,22,28,29,34,35] Lesions are variable among affected animals but may include alopecia, hyperkeratosis, dermatitis, scleroderma, conjunctivitis, orchitis, testicular atrophy, and granulomatous inflammation, mineralization, and/or fibrosis associated with degenerate cysts in any organ.[1,8,9,22,28,29,35] Historically, *Besnoitia* has been considered nonpathogenic in opossums, but recent reports suggest that inflammation, although generally mild, may be more prevalent than was previously acknowledged.[13,16,25] This article describes gross and histologic lesions associated with besnoitiosis in four Virginia opossums with molecular identification of the causative agent.

From the Athens Veterinary Diagnostic Laboratory, College of Veterinary Medicine, University of Georgia, Athens, Georgia 30602, USA (Ellis); Department of Small Animal Medicine and Surgery, College of Veterinary Medicine, University of Georgia, Athens, Georgia 30602, USA (Mackey, Moore, Divers, Hensel, Accola); Department of Pathology, College of Veterinary Medicine, University of Georgia, Athens, Georgia 30602, USA (Carmichael, Gottdenker); Southeastern Cooperative Wildlife Disease Study, Department of Population Health, College of Veterinary Medicine, University of Georgia, Athens, Georgia 30602, USA (Brown, Keel, Shock, Yabsley); and D. B. Warnell School of Forestry and Natural Resources, University of Georgia, Athens, Georgia 30602, USA (Shock, Yabsley). Correspondence should be directed to Dr. Ellis (aellis@uga.edu).

CASE REPORTS

Case 1: An adult male opossum was presented to the University of Georgia Veterinary Teaching Hospital in mid-November after being attacked by a dog. The opossum weighed 3.4 kg, was bright and alert, and had several lacerations consistent with a dog attack. Numerous 1–3-mm, firm, white nodules were present in the eyes and throughout the skin (Figs. 1 and 2). Ophthalmic examination localized lesions to the third eyelid, conjunctiva, and iridal stroma of the right eye (OD) and lid margins, conjunctiva, third eyelid, and iridal stroma of the left eye (OS). A skin biopsy confirmed numerous protozoal cysts, consistent with *Besnoitia*, within the dermis, rarely surround-

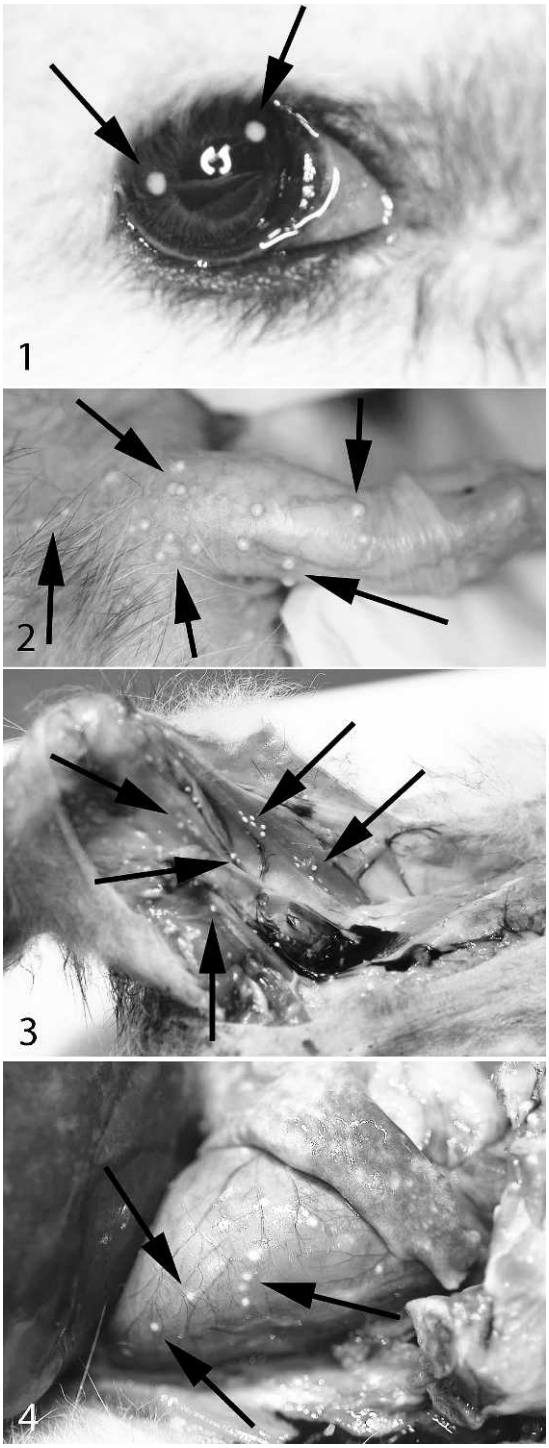

Figure 1. Eye, Case 1. Two protozoal cysts are visible on the iris (arrows). **Figure 2.** Penis, Case 1. Numerous protozoal cysts (arrows) are present on the penile sheath and the adjacent skin. **Figure 3.** Rear leg, Case 1. Clusters of protozoal cysts (arrows) are present in the muscles and fascia of the right thigh. **Figure 4.** Heart, Case 1. Several protozoal cysts (arrows) are noted on the surface of the heart.

ed by macrophages, a few plasma cells, and scattered lymphocytes. Fecal examination also identified multiple nematode species. The opossum was treated with trimethoprim sulfa (40 mg/kg per os q. 12 hr for 2 wk), fenbendazole (40 mg/kg per os q. 24 hr for 3 d), and ivermectin 1% (0.4 mg/kg SQ q. 24 hr for 3 d) and was sent to a licensed rehabilitator. A recheck examination 2 wk later identified resolution of lacerations but continued presence of ocular and cutaneous cysts. Additionally, a sheet of fibrin was associated with the corneal cysts in the right eye. Abdominal and ocular ultrasound revealed mineralized hyperechoic nodules in the kidneys and organized echogenic structures in the anterior and posterior chambers OD. An additional course of trimethoprim sulfa, ivermectin, and fenbendazole was prescribed, with the previously described dosing regimen. Over the next 6 mo, the opossum was clinically re-evaluated several times. Throughout this period, cysts were continuously present in the eyes and skin, although position and number of cysts were variable, with an initial increase followed by gradual decrease in cyst number. Over time, lesions in the lid margins, third eyelids, and conjunctiva resolved followed by resolution of corneal and anterior chamber lesions. Only iridal and nonocular cutaneous lesions were noted on examination 1 mo prior to death. In late June, the opossum was found dead and was submitted for necropsy to the Athens Veterinary Diagnostic Laboratory. On gross examination, the opossum was in good body condition. Numerous 2–3-mm diameter, raised, firm, white nodules were present on the inner aspects of the pinnae, around the mouth, and throughout the skin of the thorax and abdomen. Similar nodules were noted in the eyes, tongue, hard palate, diaphragm, lungs, kidneys, subcutis, fascia, skeletal muscle (Fig. 3), and heart (Fig. 4). Ecchymotic hemorrhages were present on the serosa of the stomach and ileum, and very firm fecal material was present in the cecum. Histologically, there was marked, multifocal mineralization of myocardial fibers (Fig. 5). Myocardial fibers were frequently separated by small numbers of lymphocytes, plasma cells, neutrophils, and macrophages that were sometimes associated with foci of mineral. In addition, there were occasional, 500–800-μm parasitic cysts within the myocardium (Fig. 5). These were surround-

ed by a thick (10–20 μm), hyaline capsule and contained myriad, teardrop-shaped bradyzoites. Some cysts contained or were surrounded by mineral or small numbers of inflammatory cells, whereas others incited no reaction. Additional foci of mineralization were noted in adrenal glands, lip, renal cortex, skeletal muscle (tongue), gastric serosa, and tunica muscularis, splenic capsule, and tunicas media and intima of blood vessels in the lungs, spleen, and stomach. With the exception of gastric and vascular lesions, mineralization was usually associated with degenerate parasitic cysts. Parasitic cysts similar to those described in the heart were present, either intact or in varying stages of degeneration, in the renal cortex (Fig. 8), adrenal glands, lymph node, lip, and skeletal muscle of the tongue, retrobulbar skeletal muscle and adipose tissue, choroid, and iris. Focally extensive, multifocal, and coalescing aggregates of fibrillar to amorphous, pale eosinophilic material interpreted to be amyloid separated or expanded cords of hepatocytes, splenic parenchyma (Fig. 6), pulmonary arterial wall, adrenal glands primarily along the corticomedullary junction, lymph node, and renal glomeruli and interstitium. In kidneys, there was scattered hypertrophy of mesangial cells, and glomerular capillaries multifocally contained increased numbers of neutrophils. Many proximal tubules were dilated and contained proteinic casts, oxalate crystals, macrophages, sloughed epithelial cells, and occasional erythrocytes or hemoglobin crystals. Tubular epithelium was variably attenuated, and some epithelial cells contained brown granular pigment. The interstitium was expanded by small numbers of lymphocytes, plasma cells, and neutrophils with variable fibrosis. In the eye, the vitreous contained a thick, fibrinous membrane with rare neutrophils, lymphocytes, and plasma cells. The cortex of the anterior, posterior, and equatorial lens contained Morgagnian globules and bladder cells. The retina immediately adjacent to the choroidal cyst was detached and necrotic. In a section of lip, there was mineralization of small numbers of individual myofibers associated with two cysts. One appeared inactive (Fig. 7), but a small rim of mineral was present along the edge of the second cyst and the entire cyst was surrounded by neutrophils and eosinophils with few mononuclear cells. Large numbers of bacteria and yeasts were noted in the keratin along the skin surface, and there were few profiles of sarcoptiform mites (Fig. 7). Additional gross and histologic changes included mild pulmonary alveolar expansion by small to moderate numbers of foamy macrophages and neutrophils, mild pulmonary medial arterial inflammation composed of lymphocytes, plasma cells, neutrophils, and macrophages, and presence of approximately 10–20 gastric nematodes.

Case 2: An adult, female opossum was found trembling and uncoordinated in May. The animal was euthanized and submitted to the Southeastern Cooperative Wildlife Disease Study (SCWDS) for necropsy. The animal was in good nutritional condition and weighed 1.98 kg. Multiple, 1–3-mm, firm, white nodules were present in the heart, kidneys, and skeletal muscle. Histology revealed multifocal areas of necrosis and inflammation, ranging from minimal to marked, in the heart and kidney, interpreted to represent degenerate protozoal cysts. These foci consisted of central areas of necrosis and mineralization surrounded by fibroblasts and epithelioid macrophages with fewer lymphocytes and plasma cells and rare neutrophils. Bradyzoites were visible in some cysts. The renal cyst had a thick, hyaline wall with a focal defect where small numbers of epithelioid macrophages, fibroblasts, and neutrophils and large numbers of lymphocytes and plasma cells infiltrated and abutted the cyst. Additional findings included mild multifocal eosinophilic and granulomatous pneumonia and gastric nematodes.

Case 3: An adult female opossum was found in March walking in circles with no apparent fear of humans. The animal was euthanized and submitted to the SCWDS for necropsy. On gross examination, the opossum was moderately thin and weighed 3.4 kg. Multifocal, 2–3-mm, firm, white nodules were noted in the subcutis, abdominal serosa, skeletal muscle, spleen, adrenal glands, tongue, kidney, and myocardium. Myocardial nodules were surrounded by pale streaks. Histologically, heart, lungs, adrenal glands (Fig. 9), intestinal musculature, and skeletal muscle had multifocal, large, round, protozoal cysts with thick, hyaline walls and numerous bradyzoites. Intracardiac cysts were associated with severe, multifocal mineralization, degeneration of cardiomyocytes, and interstitial infiltrates consisting of numerous lymphocytes, plasma cells, and macrophages with fewer neutrophils and eosinophils. Additional findings included pulmonary edema, congestion, and hemorrhage with intralesional nematodes and over 200 pink–white gastric nematodes. Liver, kidney, spleen, lymph nodes, intestines, and brain were severely autolyzed, precluding detailed evaluation.

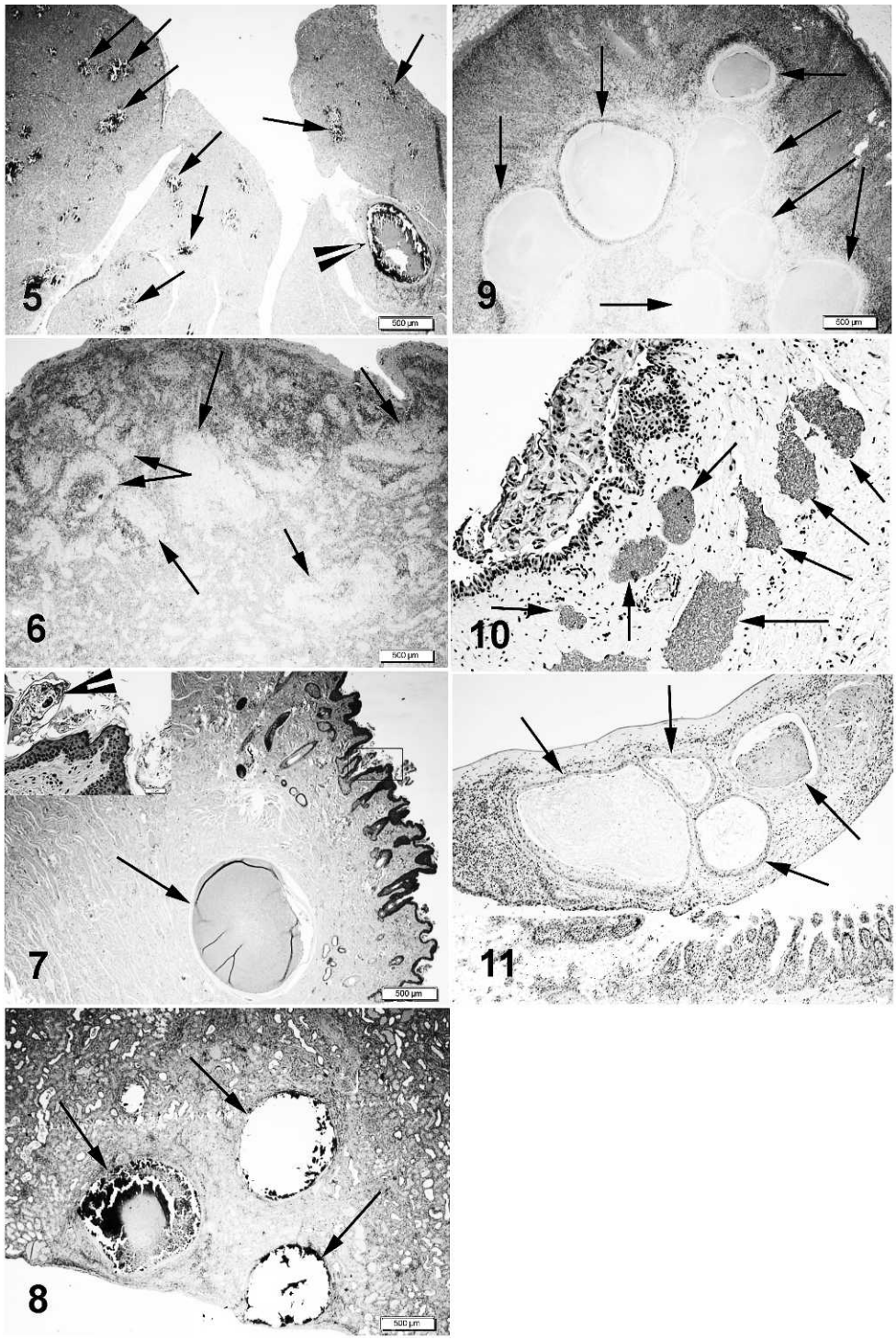

Figure 5. Heart, Case 1. Numerous foci of mineral (arrows) replace myocardial fibers and there is a single protozoal cyst (arrowhead) with peripheral mineralization. 20×, H&E stain. **Figure 6.** Spleen, Case 1. Coalescing aggregates of pale, eosinophilic material interpreted to be amyloid (arrows) are present in the parenchyma, often surrounding lymphoid follicles. 20×, H&E stain. **Figure 7.** Skin, Case 1. A quiescent protozoal cyst (arrow) is present in the dermis. 20×. Inset: A sarcoptiform mite (arrowhead) is present on the skin surface and numerous

Case 4: An adult female opossum was trapped in June during a routine tick surveillance study. The animal was in poor nutritional condition, did not respond to visual stimuli, and had old fractures in both hind feet. Because of its poor condition and lack of vision, the animal was euthanized and submitted to the SCWDS for necropsy. On gross examination, the opossum was emaciated and weighed 1.0 kg. Purulent material exuded from a 1-cm tear in the left cornea, and both eyes were tan and opaque. Numerous, 1–3-mm, firm, white, nodules were present in the skin and subcutis, and visceral organs contained numerous, multifocal to coalescing, tan, caseous, 1-mm– to 2-cm–diameter nodules. Histologically, the left eye was shrunken (phthisis bulbi) with corneal fibrosis and marked suppurative, lymphoplasmacytic, and granulomatous panopthalmitis. Erosive/ulcerative keratoconjunctivitis with superficial bacterial colonies and mild suppurative and lymphoplasmacytic uveitis were noted in the right eye. Many free zoites were present within lymphatic vessels (Fig. 10). Protozoal cysts 150–200 μm in diameter with a thick (10–20 μm), hyaline wall and numerous bradyzoites were noted in the conjunctiva OS and corneal stroma OD (Fig. 11). Cysts were surrounded by a zone of macrophages, plasma cells, and lymphocytes. Additional findings included pulmonary edema, fibrinosuppurative alveolitis with few cocci, and multifocal subpleural lipid pneumonia with mesothelial hypertrophy. Multifocal (random) areas of necrosis effaced approximately 50% of the hepatic parenchyma. A single bradyzoite-filled cyst was present in the adrenal medulla and was surrounded by a thin zone of compressed chromaffin cells, eosinophils, and fewer neutrophils. The renal interstitium, particularly the corticomedullary junction, was infiltrated by neutrophils and eosinophils with fewer macrophages and plasma cells. In severely inflamed areas, there was moderate loss of renal tubules, and remaining tubules had attenuated epithelial cells. Mild, diffuse infiltrates of neutrophils were present in the inner circular and outer circular muscle layers of the urinary bladder. Several organs, including adrenal glands, gastrointestinal tract, liver, and kidneys contained multiple foci, interpreted to be degenerate *Besnoitia* cysts, consisting of an amorphous eosinophilic core with variable hemorrhage and mineralization surrounded by fibrosis and pyogranulomatous inflammation. Additional findings included a closed fracture of the right tibia surrounded by extensive soft tissue hemorrhage and large numbers of nematodes, 0.1–0.3 cm in diameter, embedded in the wall and extending into the lumen of the stomach and proximal small intestine. Histologically, nematode cross sections were surrounded by neutrophils, eosinophils, macrophages, and occasional giant cells mixed with necrotic cellular debris and bacteria. One nematode in the muscularis of the stomach was surrounded by fibrosis and necrotic debris with associated necrosis and inflammation extending transmurally and along the serosal surface. Culture of the right eye contents yielded growth of multiple bacterial species.

Molecular characterization

Polymerase chain reaction (PCR) targeting the 18S rRNA gene was initially performed with the use of DNA extracted from cultured parasites harvested from one of the tissue cysts by a previously described protocol.[36] PCR was later performed with paraffin-embedded tissues from Cases 2–4 with the following protocol. The internal transcribed spacer (ITS)-1 region was amplified from paraffin-embedded tissues that contained *Besnoitia* cysts from Cases 2 and 3 with the use of a nested PCR protocol.[3] DNA was extracted from tissues with the use of previously described but modified protocols.[10,21] Briefly, paraffin shavings (20 μm) were digested in xylene overnight at 55 C. Excess xylene was decanted and the tissue was washed two times with 100% ethanol. Dried tissues were rehydrated in 100 μl of phosphate-buffered saline and digested with proteinase K (Qiagen, Germantown, Maryland 20874, USA) at 60 C for 72 hr (with 20 μl of proteinase K added every 24 hr) with frequent vortexing. DNA was extracted with the Qiagen DNA Purification Kit (Qiagen) in accordance

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bacteria and yeast are present in the keratinic debris. 200×, H&E stain. **Figure 8.** Kidney, Case 1. Three protozoal cysts (arrows) with varying degrees of mineralization are present in the renal cortex. 20×, H&E stain. **Figure 9.** Adrenal gland, Case 3. Numerous protozoal cysts (arrows) are present in the cortex and medulla. 20×, H&E stain. **Figure 10.** Skin, Case 4. Clusters of zoites are present within several lymphatic vessels. (arrows) 100×, H&E stain. **Figure 11.** Eye, Case 4. Multiple protozoal cysts (arrows) expand the cornea and are surrounded by an inflammatory infiltrate. 400×, H&E stain.

with the manufacturer's protocol for tissues. Attempts to amplify DNA from the paraffin-embedded tissues from Case 4 failed, likely due to lengthy fixation in formalin prior to being embedded.

The above protocols yielded a sequence of 764 bp from Case 1 (GenBank accession no. GU060623) that was most similar to *Besnoitia jellisoni* (99.6%), *Besnoitia besnoiti* and *Besnoitia tarandi* (98.8%), and *Besnoitia bennetti* (98%). The sequence also had 99.6% similarity to *Besnoitia akodoni*; however, the available sequence of *B. akodoni* was much shorter than for *B. jellisoni* (764 vs. 725 bases). At the time, there were no *B. darlingi* 18S rRNA sequences in GenBank for comparison, but it was assumed that the *Besnoitia* in our case was *B. darlingi*. No other *Besnoitia* species has been reported from Virginia opossums and cysts were histologically consistent with *B. darlingi*.[16] Subsequently, two morphologically similar cysts from Cases 2 and 3 were confirmed to be *B. darlingi* based on internal transcribed spacer (ITS)-1 region sequences as described below. Excluding short regions of the 18S (52 of 53 bp similar to *B. darlingi* [AF489696]) and 5.8S rRNA (57 of 59 bp similar to *B. darlingi*) that flanked the ITS-1 region, the 241 bp of ITS-1 sequence from Case 3 was 100% identical to *B. darlingi* (AF489696). The accession number for the partial 18S rRNA, complete ITS-1, and partial 5.8S rRNA sequence is HQ163919. The sequence from Case 2 was identical to the sequence from Case 3 except for the incorporation of two nucleotides (M) at base 72 instead of a C. The next most similar ITS-1 sequence was from *Besnoitia oryctofelisi* (AY182000) with 240 of 241 bp (99.6%) identical as well as a 2-bp insertion in *B. oryctofelisi*.

DISCUSSION

Besnoitia have previously been considered nonpathogenic in Virginia opossums, and studies in Michigan and Indiana opossums described only mild histologic lesions associated with parasitic cysts.[16,25] However, the current study suggests that in a small number of animals, infection with *Besnoitia* may cause severe disease, debilitation, or death. The true mortality rate is difficult to determine, because presence of cysts or inflammatory changes in some critical areas such as heart or eyes could predispose these animals to other more obvious causes of death such as trauma or predation. As in other species, the factors contributing to host sensitivity are unknown. One of the animals had evidence of previous trauma, one was being held in captivity, one had recently given birth, three were in poor body condition, and all had pulmonary and/or gastrointestinal parasites. All were judged to be adults, although exact age could not be determined. A previous study found a high prevalence of *Besnoitia* in adult female opossums in Indiana,[16] and three of the four animals in this report were adult females. The same study and a second study in Michigan opossums showed that prevalence was also much higher in spring and summer, and three of these four animals were submitted during that time (March–June).[16] It is uncertain whether the poor body condition of the three Indiana animals was the cause or effect of infection or if it was unrelated. However, an experimental study of besnoitiosis in cattle demonstrated that immunosuppression was required for development of severe disease,[11] and immunosuppression can be caused or exacerbated by poor nutrition. Degeneration of cysts alone apparently does not represent a pathologic event. According to Bigalke, cystic degeneration commonly occurs in equids.[2] A horse with numerous viable cysts shortly after infection had only a few calcified cysts when slaughtered 6.5 yr later, and donkeys also had an appreciable decrease in numbers of cysts after 3 yr. An unusual finding in Case 1, the only animal treated for besnoitiosis, was the presence of concurrent amyloidosis. This was not observed in any of the other three animals and has only been reported in one other Virginia opossum with besnoitiosis where amyloid was identified only in the spleen.[31] Amyloidosis has also not been associated with besnoitiosis in other species, but it is associated with chronic inflammation and has been linked to other parasitic diseases in animals including leishmaniasis, schistosomiasis, trypanosomiasis, and hepatozoonosis.[4,7,20,23,30,33] It is possible that most opossums with besnoitiosis die of other causes such as septicemia, other infections, trauma, or predation before amyloidosis can develop.

In conclusion, besnoitiosis may be a rare cause of morbidity and mortality in Virginia opossums. Although death appears to be a rare result of infection, this contradicts the traditional view that *Besnoitia* spp. are uniformly nonpathogenic in opossums. Conditions such as stress, concurrent disease, or severe malnutrition may be predisposing factors. Additional molecular characterization is needed to confirm that *B. darlingi* is the only *Besnoitia* sp. utilizing opossums as intermediate hosts.

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