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Department of Veterinary Pathology
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Conference Moderator:
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CASE I: 072188-03 (AFIP 3153650).

Signalment: Adult, male cynomolgus macaque (*Macaca fascicularis*).

History: This animal was part of a research colony of macaques at the U.S. Army Research Institute of Infectious Diseases (USAMRIID). This macaque was administered an experimental vaccine against ebolavirus. Twenty-eight days after vaccination, it was challenged with ebolavirus and it survived the viral challenge. This monkey was euthanized at the end of the study and then it was submitted for a complete necropsy.

Note: The viral challenge study and necropsy were performed under biosafety level 4 (BSL-4) conditions. The research was conducted under an Institutional Animal Care and Use Committee approved protocol in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the *Guide for the Care and Use of Laboratory Animals*, National Research Council, 1996. The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Gross Pathology: No lesions were noted grossly.

Histopathologic Description: There are two sections of tissue on the slide: 1) nasal vestibule (including haired skin and nasal mucosa) and 2) lip (including haired skin, oral mucosa, and mucocutaneous junction). Each of these tissues is described separately below.

Nasal vestibule: There are multifocal and coalescing infiltrates of moderate numbers of lymphocytes, plasma cells, and fewer eosinophils and macrophages within the lamina propria and the submucosa. These inflammatory cell infiltrates usually have a perivascular orientation and often extend into and disrupt the vessel walls. The deep dermis adjacent to the submucosa also has multifocal perivascular infiltrates of similar, but fewer, inflammatory cells. There is multifocal moderate orthokeratotic hyperkeratosis of the mucosal epithelium accompanied by aggregates of keratinized debris and numerous coccoid and bacillary bacteria along the luminal surface of the epithelium. Multifocally, infiltration of the epithelium by low numbers of neutrophils is also present.

Lip: There are multifocal infiltrates of low to moderate numbers of lymphocytes and fewer plasma cells within the submucosal labial glands, often accompanied by atrophy and loss of glandular cells.

Contributor's Morphologic Diagnosis: 1. Nasal vestibule: Multifocal and coalescing lymphoplasmacytic and eosinophilic rhinitis, moderate to marked, with mucosal hyperkeratosis and perivascular dermatitis.
2. Lip: Multifocal lymphocytic labial gland adenitis, mild.

Contributor's Comment: The location and nature of the lesions present in the nasal vestibule are consistent with those that can occur in cynomolgus and rhesus macaques infected with nematode parasites in the genus *Anatrichosoma*. In some of the histology slides from this case, sections of nematodes are present in the submucosa of the nasal vestibule. Most of these are located within the lumen of a dilated venule (or perhaps lymphatic vessel); however, one section appears to be in fibrous connective tissue and is surrounded by inflammatory cells. In some of the sections of the worm (or worms) in the vessel lumen, a stichocyte is adjacent to and partially surrounds the esophagus;(2,4) the presence of a stichosome esophagus is a distinguishing feature of trichenelloid nematodes,

which include species in the genera *Trichinella*, *Trichuris*, *Capillaria*, *Trichosomoides*, and *Anatrichosoma*.(2) Another feature of this group of nematodes is the presence of one or more hypodermal bands;(2,4) the parasites in this monkey have two such bands, which is consistent with *Anatrichosoma* spp.(2) In some of the intravascular worm sections, a testis containing developing spermatocytes is present.

Two species of *Anatrichosoma* have been described in macaques, *A. cynamolgi* and *A. cutaneum*, and each species can infect both cynomolgus and rhesus macaques.(1,3,8,10) The first of these parasite species discovered, *A. cutaneum*, was initially described in association with significant inflammatory lesions in the skin and subcutis of the hands and feet of rhesus macaques. However, these were apparently aberrant locations for the parasites;(1) the mucosa and submucosa of the nasal vestibule is the normal site of parasitism for both species of worms,(1,3) and infections in this location have not been associated with clinical signs or gross lesions. Differentiation of the two *Anatrichosoma* species is most reliably accomplished by examination of unfixed eggs and/or intact worms.(3,10)

Surveys of wild-caught macaques have indicated that the prevalence of infection in some free-ranging populations can range as high as 48%.(5) In one report concerning an experimental inhalation toxicity study, the parasites and/or nasal inflammation consistent with *Anatrichosoma* sp. infection were detected in 27 of 32 (84%) juvenile rhesus macaques.(6)

The full life cycle of these parasites is unknown. Mature female worms are located within the stratified squamous epithelium of the nasal vestibule where they are associated with epithelial hyperplasia and hyperkeratosis and varying degrees of inflammation of the epithelium and underlying tissues.(1,6,7) The female worms and released eggs are often present within intra-epithelial "tunnels" made by the worms. Immature parasites and mature males are usually located in the lamina propria and submucosa and are often intravascular (as in this case).(1,3,7,10) It is postulated that the male worms migrate up to the epithelial layer for mating and then return to the deeper tissues afterwards.(7) Eggs may be released directly by the females into the nasal vestibular lumen or reach the lumen from the intra-epithelial tunnels during epithelial desquamation.

There have been limited and unsuccessful attempts to experimentally infect macaques by direct transmission of the eggs.(10) This has led to speculation that one or more intermediate hosts are required.(9)

Although fecal samples from *Anatrichosoma*-infected monkeys may contain the parasite eggs, examination of nasal swabs for presence of eggs has been shown to be a more reliable method for ante mortem detection of parasitized individuals.(5)

The inflammation present in the labial glands of this case is mild; this is a common, incidental finding in macaques and is unlikely to be associated with the ebolavirus challenge or the nematode parasitism. At our institution, we routinely include a section of lip in the histology block with the nasal vestibule.

Note: Opinions, interpretations, conclusions, and recommendations above are those of the author and are not necessarily endorsed by the U.S. Army or Department of Defense.

AFIP Diagnosis: 1. Haired skin and nasal vestibule: Rhinitis, lymphoplasmacytic, histiocytic, and eosinophilic, multifocal to coalescing, moderate, with epithelial hyperplasia, orthokeratotic and parakeratotic hyperkeratosis, spongiosis, and perivasculitis.
2. Mucocutaneous junction, lip, minor salivary gland: Sialoadenitis, interstitial, lymphoplasmacytic and histiocytic, chronic, multifocal, mild.

Conference Comment: Unfortunately, of the 195 slides produced for this WSC submission, only one contained sections of the nematode. The contributor, who also moderated this conference, remarked that this case accurately reflects reality, i.e. the nematodes are often scarce in histologic section, and the diagnosis must often be made presumptively based on telltale inflammation in the nasal vestibule. In the absence of a nematode in the submitted sections, conference participants considered a hypersensitivity reaction most likely, and suggested a number of potential etiologies, including an inhaled environmental allergen, arthropod bite, or intranasal vaccine. The contributor provides a detailed review of anatrichosomiasis.

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CASE II: R04-43 (AFIP 2948651).

Signalment: Adult, female crocodile (*Crocodylus niloticus*).

History: This crocodile was kept in a local zoo for over 2 years and was listless and anorexic for 5 weeks.

Gross Pathology: All lung lobes contained multiple, 0.5-1.0 cm diameter, irregularly-shaped, well-defined, firm, homogenous white to yellow nodules. Several firm, large nodules, 2.0-2.5 cm in diameter, white to yellowish in color, surrounded by dark red foci, were found in some part of the lungs. Visceral surfaces in the lung and pleural surfaces appeared dull and rough with a yellowish fibrous appearance. The liver and spleen were enlarged and cut surfaces revealed several gray to pale nodules.

Laboratory Results: *Escherichia coli* and *Aeromonas* sp. were isolated from the lungs and liver.

Histopathologic Description: Microscopic examination of the lung consists of a hemorrhagic, necrotizing bronchopneumonia with massive aerial mycelial growth within the airways. These affected airways are filled with numerous degenerated heterophils admixed with macrophages, necrotic debris, and bacillary colonies. The fungal organisms contain 3-5 µm chains of conidia, 4-7 µm dichotomous-branching, septate fungal hyphae. Airways are markedly dilated and the mucosal epithelium is extensively ulcerative with hemorrhage, many heterophils and macrophages are invading into the adjacent lung parenchyma. In these pneumonic areas, inflammation is associated with the appearance of numerous birefringent crystals with radiating spokes; the morphology of these crystals is suggestive of calcium oxalate. Histochemistry reveals many PAS-positive fungal hyphae scattered throughout the necrotic foci of lungs, liver, and spleen, primarily along the margins of the necrotic lesion.

Contributor's Morphologic Diagnosis: Lung: Bronchopneumonia, necrogranulomatous and hemorrhagic, severe, subacute to chronic, multiple, with intralesional fungal hyphae, oxalate crystals, necrotic vasculitis, and pleuritis, crocodile (*Crocodylus niloticus*), reptile.

Contributor's Comment: Clinically, oxalate is produced by a variety of fungi, including saprophytic (e.g. *Aspergillus niger* and *A. flavus*) and phytopathogenic species, and other sources including methoxyflurane, ethylene glycol, large doses of ascorbic acid (vitamin C), primary hyperoxaluria (humans, cats, and tibetan spaniels), pyridoxine (vitamin B_6) deficiency, and plants (e.g. *Halogeton glomeratus*, *Sarcobatus vermiculatus*, *Rheum rhaponticum*, *Oxalis cernua*, *Rumex* sp., Russian vine, etc.). Several pathways have been described for oxalic acid production, but the mechanism of oxalate crystal production is not known. In *Aspergillus niger*, the organism apparently possesses oxaloacetate acetylhydrolase (OAH), one of the enzymes of the tricarboxylic acid cycle, and degrades oxaloacetate to oxalate by this route (i.e. oxaloacetate + $H_2O \rightarrow$ oxalate + acetate). In humans, similar

morphological findings have been reported, with often localized deposition of calcium oxalate crystals around a pulmonary *A. niger* fungal ball.(1-3,5,6)

Aspergillus, a ubiquitous environmental organism, is an opportunistic pathogen in mammals and birds. *Aspergillus fumigatus* is the most commonly reported cause of aspergillosis in animals, whereas *A. flavus*, *A. terreus*, *A. nidulans*, and *A. niger* are incriminated less frequently. In animals, localized deposition of massive numbers of calcium oxalate crystals in tissues has been reported in association with *Aspergillus* infections.(5,7-9) This is the first report of pulmonary oxalosis in a crocodile.

AFIP Diagnosis: Lung: Pneumonia, necrohemorrhagic, multifocal to coalescing, severe, with pleuritis, myriad fungal hyphae, and abundant anisotropic crystalline material (oxalate crystals).

Conference Comment: The GMS method highlights the fungal conidia and is helpful in demonstrating the other hyphal characteristics described by the contributor; while these features, in combination with oxalate crystal formation, are certainly suggestive of aspergillosis, the results of confirmatory testing (e.g. fungal culture) would be particularly valuable for solidifying the specific etiologic diagnosis. Furthermore, while the morphology of the prismatic crystalline material is consistent with oxalate crystals, histochemical stains (e.g. Yasue's sliver nitrate-rubeanic acid), though not performed, may be employed to definitively identify oxalate crystals.(5)

In domestic species, oxalate crystals are more commonly encountered in the renal tubules due to ethylene glycol toxicosis in cats and dogs, or intoxication with one of the oxalate-containing plants listed above in sheep and cattle. Interestingly, the rumen microflora have some capacity to degrade oxalate to bicarbonate and carbonates, conferring sheep and cattle with a degree, albeit limited, of resistance to oxalosis; yet, remarkably, horses are far more resistant to oxalate-induced nephrosis. Because oxalate chelates calcium, hypocalcemia is a characteristic finding in both ethylene glycol toxicosis and oxalate toxicosis.(4)

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CASE III: 0408061 (AFIP 2940154).

Signalment: Mature female red-tailed boa constrictor (*Boa constrictor*).

History: This snake became listless, inappetant, and died approximately one month after birthing. It had regurgitated recently. One other snake (also a red-tailed boa) also died recently.

Gross Pathology: This boa was in good nutritional condition and had ample internal body fat stores. No gross lesions were seen.

Contributor's Morphologic Diagnosis: Inclusion body disease of boids, with widespread intracytoplasmic inclusion bodies in epithelial cells of liver hepatocytes and biliary epithelial cells and hepatocellular degeneration and necrosis.

Contributor's Comment: Inclusion body disease of boid snakes has been recognized for the past 30 years, and is named for the characteristic intracytoplasmic inclusion bodies seen in a variety of epithelial cells. These include epithelial cells of the pancreas, renal tubules, gastrointestinal tract, respiratory tract, hepatocytes, biliary epithelium, thyroid follicular cells, and neurons of brain and spinal cord.(4) All tissues involved are reported to have varying degrees of degenerative changes, including vacuolation, cellular collapse and necrosis. Meningoencephalitis has been reported in both boas and pythons; however, the severity of both the neural histopathologic changes and clinical signs is much higher in Burmese pythons than in boa constrictors.(4)

Workers at the University of Florida found this disease to be caused by a retrovirus through the use of transmission electron microscopy. Further characterization efforts conducted demonstrated reverse transcriptase activity. Western blot analysis of viruses from different snakes was also done; these different isolates from different snakes were found to be similar.(1)

Clinical signs are quite variable. Regurgitation and signs of central nervous system (CNS) disease are commonly seen in boa constrictors. Stomatitis, pneumonia, undifferentiated cutaneous sarcomas, and lymphoproliferative disorders have all been seen. Burmese pythons generally show signs of CNS disease without manifesting any other clinical signs; regurgitation is not seen in Burmese pythons.(4)

This snake was one of several in this household that died with similar clinical presentations and pathologic findings. There is no treatment, and the disease is highly contagious and always fatal. As the disease seems to be occurring with increasing incidence, it is imperative that all new boids brought into a collection be quarantined from acquisition for at least 3-6 months, and that appropriate precautions be taken when visiting other collections, pet stores, expositions, and swap events.

Although slides submitted from this snake for the conference were all from the liver, similar inclusion bodies were also seen in most of the tissues cited above.

AFIP Diagnosis: 1. Liver: Hepatocellular degeneration, diffuse, moderate, with scattered single hepatocellular necrosis, and many hepatocellular and biliary epithelial intracytoplasmic eosinophilic inclusion bodies.
2. Liver: Hepatitis, necrotizing, random, acute, multifocal, moderate, with colonies of coccobacilli.

Conference Comment: Conference participants noted the characteristic eosinophilic intracytoplasmic inclusion bodies both in hepatocytes and biliary epithelial cells. Additionally, scattered randomly throughout the liver are foci of lytic necrosis with colonies of coccobacilli, which participants ascribed to embolic showering resulting from terminal sepsis, thus accounting for the lack of associated inflammation.

Despite an abundance of research on this important entity, fulfillment of Koch's postulates remains elusive, and the cause of inclusion body disease (IBD) is still enigmatic. As noted by the contributor, a retroviral etiology has been strongly suspected, and clinicopathologic evidence (e.g. stomatitis, lymphoproliferative disorders, etc.) supports this conclusion. Moreover, retroviruses have been isolated from various species of snakes with IBD; however, a causal role has not been established, and it remains possible that retroviruses play only an incidental role in IBD. Similarly, reoviruses and adenoviruses have also been isolated from snakes with IBD, and may play causal and/or incidental roles in the disease. Notably, electron microscopy demonstrates that IBD inclusions are nonviral, and consist of radio-dense round particles that accumulate at the periphery of the inclusion; particles are composed of a 68-KDa protein deposited by polyribosomes.(2)

As noted by the contributor, epidemiologic evidence suggests that IBD is indeed contagious, and quarantine measures are therefore warranted. Antemortem diagnosis is achieved by biopsy examination of the liver or kidney, or the esophageal tonsil, in which inclusions are generally present in the overlying epithelial cells.(2) In a recent study, investigators readily identified inclusions in the leukocytes of boas with IBD using concentrated buffy coat examinations, and hence recommended the screening technique as a less invasive alternative to biopsy; however, the technique may not be applicable in pythons, in which the distribution of inclusions is often limited to the central nervous system.(3)

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CASE IV: 60848 (AFIP 3134333).

Signalment: Adult, female, wild-caught Giant Pacific octopus (*Octopus (Enteroctopus) dofleini*).

History: Three Giant Pacific octopuses, *Octopus (Enteroctopus) dofleini*, wild-caught in Alaska and held in captivity, died or were euthanized following periods of decreased appetite and lethargy, despite treatments for possible septicemia and heavy metal exposure. This particular wild-caught female came in October 2006 and spawned 20 April 2008. She continued to eat, interact with the environment, and behave normally until found moribund 4 December 2008, requiring euthanasia. Lesions were similar in all three giant octopus submissions.

Gross Pathology: Multiple gill biopsies were submitted in formalin and 95% ethanol.

Histopathologic Description: Gills: There are abundant piriform structures lining up along the apical surface of columnar gill epithelium. The parasites extend slender structures connecting them to apical aspects of the epithelium. The submucosa is diffusely and variably expanded by numerous degenerate and viable hemocytes with abundant granular eosinophilic cytoplasm and reniform-to-bilobed nuclei. Within the inflammatory hemocytes, and often extracellularly, is variably-sized spherical, amphophilic, pink-to-olive brown, refractile material. Individual inflammatory cells are observed transmigrating through the crowded tall columnar pseudostratified epithelial cells and occasionally coalescing into small aggregates of exudated hemocytes. There is some epithelial loss with parasites adhered to areas of disruption; adjacent degenerate to necrotic epithelial cells are vacuolated or collapsed with nuclear pyknosis. There are multifocal areas of increased clear space between haphazardly arranged bundles and individual connective tissue fibers of the submucosa (edema).

Contributor's Morphologic Diagnosis: Gills: Inflammation, subacute, hemocytic, diffuse, severe with epithelial necrosis (branchitis).

Etiologic Diagnosis: Necrotizing branchitis with intralesional apical piriform protozoa consistent with *Ichthyobodo.*

Contributor's Comment: Histopathological findings showed extensive gill degeneration and necrosis and hemocytic inflammation associated with a heavy infection of *Ichthyobodo*-like flagellates. The gills had a large number of the piriform protozoal structures queued up along the apical margin of the gill epithelium, and attended by variable submucosal hemocytic inflammation.

Samples of gills tissues were processed for genomic DNA extraction, PCR amplification, and cloning and sequencing of the rRNA genes; phylogenetic analysis was conducted on the SSU, D1-D3 and D8-D10 LSU rDNA sequences. Gene sequences were deposited in GenBank. Phylogenetic analysis indicated that the *Ichthyobodo* species identified in this study was most closely related to those from freshwater fish.

Ultrastructural features were similarly characteristic of *Ichthyobodo* species. Scanning electron microscopy showed that the flagellates were flattened pyriform to trapezoidal, approximately 6-10 µm long and 3-6 µm wide. In some regions the infection was so dense that the gill surfaces were completely covered with the flagellates. Flagellates were attached to the host cell's cytoplasm by a narrow cytostome via a crateriform puncture through the smooth mucous covering of the gill epithelium. Transmission electron microscopy revealed two unequal-width flagella

lying in a U-shaped flagellar pocket, microtubules and striated fibers surrounding the flagellar pocket, and radiating fibres lying in a semi-circle around the cytostome primordial. The spherical nucleus had a large central nucleolus and peripheral chromatin, and the cytoplasm also contained rough endoplasmic reticulum, and mitochondria. There was an attachment plate anchoring the flagellate to the epithelial cell, and the cytostome process, reinforced with fibrillar structures, passed though the plate into the cytoplasm of the host cell.

Bodonid flagellates, of the genus *Ichthyobodo*, have long been recognized as significant ectoparasitic pathogens of freshwater and marine fish in aquaculture, and have been the subject of extensive research. The parasite can be found both free-swimming in the water column and attached to epithelial surfaces such as gills and skin.(6,13) The free-swimming form moves in a spiral motion with the aid of flagella. *Ichthyobodo* attaches to epithelial cells via a long, slender organelle that facilitates the ingestion of host cellular contents.(6,13) Ichthyobodiasis can impede osmoregulation and predispose to secondary infection; alternatively, *Ichthyobodo* infections are often found in association with stressful circumstances in freshwater fish.(6,13) In contrast, there are only intermittent reports of bodonids and "*Ichthyobodo*-like" flagellates from cephalopods, and their host-parasite interaction and identity have not been reported in detail.

Invertebrates, including mollusks, rely on innate immunity to combat disease, with no evidence of acquired immunity.(9) Members of the phylum Mollusca, which includes the class Cephalopoda (squids, cuttlefish, octopus and nautilus), have both cellular and humoral mechanisms of defense. Hemagluttins are the best studied and understood component of molluscan "humoral" immunity. These soluble factors, named after the agglutination of erythrocytes from other species, is thought to play a role in the recognition and opsonization of foreign material, although cell-free hemolymph is reported to have limited ability to agglutinate bacterial isolates in vitro.(3) The hemocyte is the primary component of cephalopod cellular immunity and develops from stem cells found in the white body, a multilobed leukopoietic organ located in the retrobulbar areas.(4) The role of octopus hemocytes in wound healing is well documented; a progression from skin wound infiltration, transformation of hemocytes to fibroblastic morphology and establishment of a "dermal plug" with subsequent contraction culminates in wound repair.(2) A multi-function cell with oxygen-carrying and nutrient transport capacities, the octopus hemocyte also performs phagocytosis, producing reactive oxygen species and lysozyme to destroy pathogens.(8,11) Stress and low temperature have been shown to alter the phagocytic function of hemocytes in vivo.(7) In addition to phagocytosis, hemocyte encapsulation serves as defense mechanism against foreign substances. Resident hemocytes are described in gill tissues of octopus, complicating the evaluation and characterization of potential gill pathogens.(12)

The reproductive physiology of cephalopods is interesting and enigmatic. In cephalopods including Giant Pacific octopus, endocrine secretions from the paired optic glands direct the development of senescence, characterized by loss of appetite, change in feeding behavior, loss of condition and behavioral changes that culminate in death. Hence these species of octopus have a brief lifespan of only about 3 years and inevitably die after spawning.(1) In the captive octopus of this report, it is unclear whether there is any association of senescence and enhanced susceptibility to the *Ichthyobodo* infections. Removal of the optic glands is reported to reduce senescence-associated behavior and greatly extends the lifespan of cephalopods.(14)

These observations demonstrate that *Ichthyobodo* sp. can be a significant ectoparasitic pathogen of captive cephalopods. The host-parasite interaction and parasite ultrastructure are essentially similar to those of the better known *Ichthyobodo* sp. affecting teleosts. Molecular phylogeny showed that the *Ichthyobodo* sp. from these wild-caught captive Giant Pacific octopuses was most closely related to flagellates from several species of freshwater teleosts.

AFIP Diagnosis: Gill: Branchitis, hemocytic, multifocal, moderate, with many surface epithelial-associated flagellated protozoa.

Conference Comment: The contributor provides an excellent synopsis of the entity in this mysterious species. Conference participants discussed the challenge of estimating a lesion's chronicity based solely on microscopic examination in invertebrate species. In contrast to most vertebrates, in which the composition of inflammatory infiltrates correlates fairly reliably with chronicity, to the best of our knowledge, the composition of the inflammatory infiltrate has not been reported relative to lesion chronicity in the octopus, in which the hemocyte is the primary inflammatory cell. Accordingly, participants debated the merits of including the modifier "hemocytic" in the morphologic diagnosis, with some favoring its omission on the basis of redundancy, though most agreed with the contributor that its inclusion is an appropriate enhancement to the morphologic diagnosis, most precisely reflecting the pathologic processes involved.

Because *Ichthyobodo* infection is commonly associated with stress in freshwater fish, participants speculated that senescence-associated immunosuppression may have rendered this octopus particularly vulnerable to parasitism by *Ichthyobodo*-like flagellates. Curiously, some species of *Ichthyobodo* are capable of survival both in saltwater and freshwater; for instance, *I. necator*, which survives and causes disease over a wide range of temperatures, can survive in saltwater and cause mortality in marine-adapted salmonids. Other *Ichthyobodo*-like flagellates exclusively infect marine fish.(10) *Ichthyobodo*-like flagellate infection was reported in a laboratory cultured population of California mud-flat octopus (*Octopus bimaculoides*) and two related species (*O. maya* and *O. digueti*), in which the organism was initially found on the gill, then spread over the course of the disease to involve the internal surface of the mantle cavity and the body surface, leading the authors to conclude that the gill was the initial site of infection. Gross lesions included small white foci on the dorsal surfaces of the arms and mantle; however, these may have been partly attributed to co-infection with an ancistrocomid ciliate.(5)

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