"A Chip Off the Old (Penguin) Rock"

A Clinical Approach to Respiratory Disease in African Penguins

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Introduction

The African penguin (*Spheniscus demersus*) is a temperate species of penguin that inhabits the coastline of southwestern Africa. Over the last century, wild penguin populations have experienced a sharp decline, and this species is now considered endangered according to the IUCN Red List.[8] Considerable efforts are currently being made to support breeding of this species in captive populations in coordination with the AZA Species Survival Plan for African penguins.[2] As captive populations of penguins grow and change, aspergillosis remains a primary cause of morbidity and mortality within zoos and rehabilitation centers. Aspergillosis is a mycotic disease caused by any species within the genus *Aspergillus* and most commonly involves *Aspergillus fumigatus*. Due to generally non-specific clinical signs and subsequently delayed presentation, prognosis of this disease is guarded. Prolonged treatment with a variety of antifungal agents has been attempted in these cases with some success. Prevention of aspergillosis is key to management of captive penguin populations and centers around proper husbandry, exhibit and holding air filtration and maintenance, and thorough disinfection.

History and Presentation

Chip, an approximately 3-year old male African penguin (*Spheniscus demersus*), presented to Memphis Zoo's animal medical facility on March 13, 2019 after being noted in poor body condition during handling for his annual vaccination. His keepers observed that he had become lethargic and inappetent over the week prior to vaccination. Chip had previously presented to the animal health facility on October 2, 2016 following a similar episode of lethargy and inappetence, at which time he was diagnosed with avian malaria (*Plasmodium*). He was subsequently treated, managed supportively, and rejoined his rookery successfully in February of 2017. At the time of presentation, Chip's management and preventive care regimen included

annual West Nile Virus vaccinations, salt and formulated vitamin supplements, and sulfadiazine, pyrimethamine, and folic acid capsules for malaria prevention.

Following presentation, Chip was hospitalized in an aquatic quarantine facility and monitored prior to veterinary evaluation. He was treated proactively with terbinafine (15 mg/kg orally every 24 hours) and handfed until veterinary evaluation was performed on March 18, 2019. At presentation, Chip was thin with a body condition score of 3/9, and he weighed 2.12 kilograms. He was moderately strong in hand during manual restraint, though he exhibited severe dyspnea and tired quickly. He was successfully immobilized using isoflurane gas via facemask. His vitals were within normal limits with a heart rate of 252 beats per minute and a respiratory rate of 24 breaths per minute. No murmurs or arrhythmias were appreciated on auscultation, and normal air flow was appreciated in all air sacs. The remainder of his physical exam was unremarkable. Due to his history, physical exam, and species predilection, avian malaria and aspergillosis were primary differentials for Chip's condition. Further diagnostic evaluation was required to determine the most successful form of treatment.

Diagnostic Approach

In order to guide future diagnostics, a complete blood count (CBC) was obtained while Chip was anesthetized. A manual CBC revealed a marked leukocytosis (29,854/uL) characterized predominantly by a heterophilia (77%) and a monocytosis (14%) and a normal hematocrit (48%). A blood smear was performed to examine Chip's blood for the presence of blood parasites, and no parasites were noted, making malaria less likely. Malaria could not be entirely ruled out by blood smear alone due to the lack of inherent sensitivity of this diagnostic test for avian malaria. Fresh plasma was collected and submitted to the University of Miami's Avian and Wildlife Laboratory for *Aspergillus* testing (including antibody, galactomannan, and

plasma protein electrophoresis). Results revealed an *Aspergillus* antibody value of 1.8 (moderate positive), galactomannan value of 0.2 (negative), hyperproteinemia (7.4 mg/dl), and markedly elevated α2-globulins and β-globulins (2.2 and 3.13 respectively). These results are consistent with a clinical diagnosis of aspergillosis in penguins. Due to lack of improvement with initial medical management, Chip was anesthetized a second time on March 27, 2019. A second blood smear was evaluated for the presence of *Plasmodium*, and no blood parasites were seen. Fresh plasma was submitted again to the University of Miami's Avian and Wildlife Laboratory for *Aspergillus* testing. Full-body radiographs were obtained while Chip was anesthetized and revealed a thin body condition and a moderate to severe alveolar pulmonary pattern throughout the entire right lung and portions of the left lung. His cervical air sac was also gas-dilated, indicating respiratory difficulty. Due to increased dyspnea and poor oxygenation, Chip was quickly and successfully recovered from anesthesia. Based on preliminary diagnostics findings, Chip was presumptively diagnosed with aspergillosis.

Pathophysiology

Aspergillosis refers to any form of mycosis caused by a species of mold belonging to the genus *Aspergillus*. These organisms are opportunistic saprophytes that are ubiquitous in many environments and rarely cause disease in healthy animals. However, aspergillosis is reported as a major cause of morbidity and mortality of several avian species, particularly in captive penguin populations.[10] The susceptibility of avian species to aspergillosis is considered multi-factorial and not entirely understood. Several environmental factors have been related to an increased likelihood of infection in these species, including increased environmental spore load, elevated temperature, and poor sanitation and ventilation of indoor holding facilities. Other factors, such as tetracycline or steroid administration, poor diet, exhibit crowding, concurrent illness, and

toxin exposure have also been linked to immunosuppression and subsequent mycotic infection in captive birds.[3,8,12] Respiratory anatomy and immunology are also thought to be critical components of disease development and may explain interspecies differences in innate susceptibility. Birds characteristically lack an epiglottis and diaphragm. These structures play an active role in preventing the inhalation of particulate matter into the respiratory tract and the production of a cough reflex to expel inhaled debris.[12] In contrast to the mammalian respiratory tract, which has numerous alveolar macrophages, the avian respiratory system has a much lower number of specialized respiratory macrophages named free avian respiratory macrophages (FARM).[9,13] These macrophages are innately more capable of phagocytizing pathogens that may enter the avian lung and are supported by a functional mucociliary escalator system, epithelial tight junctions, and a structured filtration system.[9] Germination of a small number of phagocytized *Aspergillus* conidia has been reported inside FARM, particularly during times of overwhelming spore exposure. This leads to subsequent degeneration and necrosis of these macrophages and promotes development of clinical disease. [13]

Aspergillus fumigatus is most commonly diagnosed in cases of avian aspergillosis with inhalation being the primary route of infection.[3,10] Other commonly implicated species include *Aspergillus flavus, Aspergillus glaucus,* and *Aspergillus nidulans*. Upon inhalation, *Aspergillus* conidia are carried to the caudal air sacs prior to entry into the lungs. This often results in infection within the caudal sacs as spores settle out of the air current. Conidia that are carried into lung parenchyma typically embed in atria and infundibula where phagocytic cells attempt to engulf conidia at the air capillaries. If reproductive spores are not successfully eliminated, fungal plaques and necrotic tissue can accumulate in lower airways resulting in respiratory compromise.[3] The development of systemic disease may occur secondary to hyphal penetration

of air sacs or hematogenous spread of phagocytized conidia by macrophages. Two primary forms of tissue involvement are recorded in literature. A granulomatous form of disease results in the development of encapsulated granulomas in aerated and non-aerated tissues. A second form of infection results in a non-encapsulated growth of hyphae primarily in aerated tissues. Mixed infections exhibiting both forms of tissue reaction have also been reported.[3]

Aspergillosis can take many clinical forms depending on spore load and distribution of disease. Respiratory aspergillosis is most common in birds, though nasal, ocular, and dermal forms have been reported. Clinical signs of respiratory aspergillosis in birds are typically non-specific, which can delay diagnosis and appropriate treatment. General signs of respiratory mycosis may include lethargy, inappetence, wasting, dyspnea, diarrhea, vomiting, or wing drooping.[3,8,12] Further diagnostics are typically required to support a clinical diagnosis of aspergillosis. Supportive hematology and biochemistry results include a leukocytosis of over 20,000 white blood cells per microliter characterized by a heterophilia and monocytosis, a non-regenerative anemia, and hyperproteinemia with an increased globulin fraction.[3,8] Immunosuppressed birds, however, may not exhibit a marked hyperproteinemia or leukocytosis.[3] Radiography may reveal signs of pneumonia or airsacculitis, which are supportive but non-specific indications of respiratory disease. Computed tomography scans may additionally be used to evaluate the extensiveness of respiratory lesions but are also non-specific. Definitive antemortem diagnosis is difficult but may be obtained by cytology or culture of samples collected via celioscopy. This procedure is not commonly performed, however, due to limited access to equipment and the fact that many patients are not considered viable candidates for prolonged anesthesia.[3] Currently, plasma protein electrophoresis (EPH) coupled with serology is

most commonly used by practitioners to support a presumptive antemortem diagnosis of aspergillosis.

The University of Miami Avian and Wildlife Laboratory offers a comprehensive assay to evaluate for the presence of *Aspergillus* in a variety of avian species. This assay includes quantitative evaluation for *Aspergillus* antibodies, a commercial assay for galactomannan antigen (a polysaccharide in the wall of *Aspergillus* species), and quantitative evaluation of plasma proteins via standardized electrophoresis. No single part of this assay is independently diagnostic. Rather, the result of each test should be interpreted alongside patient presentation and clinical signs to make a presumptive diagnosis of aspergillosis.[4] The limitations of this *Aspergillus* assay as an antemortem diagnostic tool should be considered in suspected cases of aspergillosis.[4,5] Both healthy and infected penguins are commonly found to have high levels of anti-*Aspergillus* antibody and typically are negative for galactomannan antigen detection. For this reason, practitioners rely heavily on plasma protein EPH to support a clinical diagnosis of aspergillosis and initiate therapy. Penguins infected with *Aspergillus* showed most significant changes in α2-globulins and β-globulins when compared to non-*Aspergillus* inflammatory controls. β-globulins increased to greater than 1.52 g/dL in approximately 80% of confirmed cases, and elevations of α2-globulins over 1.16 g/dL showed the highest specificity (92.2%) for aspergillosis compared to inflammatory controls. This fluctuation of α2-globulins and β-globulins is considered unique to penguin species. Evaluation of diagnostic methods has shown evidence that levels of 3-hydroxybutyrate, a fatty acid anion, are typically elevated in birds infected with aspergillosis. These elevations indicate that the body is adjusting metabolically to promote fat mobilization in response to significant illness. However, several factors may affect 3-hydroxybutyrate levels present in the blood, including age, growth period, and a history of

anorexia. It has been suggested that 3-hydroxybutyrate levels interpreted with plasma protein EPH results may increase specificity of antemortem diagnostics and provide more accurate prognostic indication.[4,5]

Treatment of aspergillosis in birds is typically challenging due to delayed identification of disease, the granulomatous nature of infection, and a lack of knowledge of the pharmacokinetics of antifungal drugs in avian species. The basis of antifungal therapy in birds is often based on practitioner preference and extrapolation from its use in other species. Topical therapy for aspergillosis following manual removal of granulomatous lesions has shown success; however, this is not practical in many cases due to the state of disease or location of granulomas within the respiratory tract. Topical therapy without granuloma removal may be employed by way of nebulization, flushing of the nasal cavity or air sacs, or surgical irrigation.[1,3] Any attempt at topical therapy should be coupled with systemic antifungal therapy.

Most commonly, systemic therapy includes oral administration of an azole with or without supplemental treatment using terbinafine or amphotericin B. Commercial itraconazole has become a mainstay in the systemic treatment of aspergillosis in birds.[1,2,3] It is important to note that compounded itraconazole has markedly different pharmacokinetics when compared to commercial itraconazole due to poor systemic absorption.[11] For this reason, compounded itraconazole is not recommended for use in penguins. Voriconazole has gained recent popularity as an effective alternative treatment option due to an increasing number of cases of itraconazole-resistant aspergillosis.[6] Care should be taken when determining the appropriate dosage for individual species of penguins as cases of voriconazole toxicity have been reported when using dosages extrapolated between avian species.[6,7] Due to this, penguins undergoing treatment with voriconazole should be monitored closely for signs of the development of toxicity, including

anorexia, paresis, vision changes, and seizure-like activity. Therapeutic drug monitoring is strongly suggested for any avian species while undergoing treatment with voriconazole.[7]

The prognosis of avian aspergillosis is guarded. Due to the non-specific nature of common clinical signs, treatment is typically initiated late in the disease process, and toxicity may limit the duration of treatment.[2] Prevention of this disease is a priority in the management of captive populations of African penguins. Husbandry protocols should be evaluated in order to limit environmental stress as much as possible. In situations associated with high stress, such as shipping or introduction to a new environment, prophylactic antifungal therapy may be indicated. Air quality should be closely monitored, particularly in indoor/outdoor exhibits. This includes maintenance of an adequate air filtration system, regular air cultures, and thorough disinfection.[2,10]

Treatment and Management

On March 27, Chip was moved to a pen in the intensive care unit and prescribed itraconazole (Sporonox, 20 mg/kg orally every 24 hours), marbofloxacin (15 mg/kg orally every 12 hours), and ceftiofur sodium (10 mg/kg subcutaneously every 72 hours) in addition to continued terbinafine therapy. Chip was also supported by assist-feeding four times per day to address his loss of body condition and ongoing inappetence.

Chip's second *Aspergillus* test results were reported on March 29 and revealed an *Aspergillus* antibody value of 1.8 (moderate positive), a comparatively elevated galactomannan value of 0.4 (negative), normal plasma protein (6.8 mg/dl) and moderately elevated α2-globulins and β globulins (1.99 and 2.89 respectively). Medical management and supportive care were continued as his condition declined. Throughout the week, he became sternally recumbent and increasingly dyspneic, and he began intermittently regurgitating. However, on April 4, Chip was

standing more often and actively vocalizing for food. He ate voluntarily and showed marked clinical improvement. Chip was subsequently moved to an indoor holding facility and given access to a small pool, and terbinafine therapy was discontinued. That day, he was restrained for venipuncture, and blood was drawn for a CBC, serum chemistry, and *Aspergillus* testing. At this time, Chip had a persistent leukocytosis (25,630/ul) characterized by 72% heterophils, 14% lymphocytes, and 6% monocytes, and his hematocrit was 42%. His aspartate aminotransferase (AST) was mildly elevated at 238; all other findings were unremarkable. No blood parasites were noted on blood smear evaluation. Chip's *Aspergillus* assay revealed an elevated *Aspergillus* antibody value of 2.0 (strong positive), a galactomannan value of 0.2 (negative), normal plasma protein (5.4 mg/dl), hypoalbuminemia (0.88), and persistently elevated α2-globulins and β globulins (1.37 and 2.19 respectively). These results illustrated mild improvement and maturation of the infection. Within approximately 48 hours of being transferred to a new holding pen, Chip became increasingly dyspneic and sternally recumbent again. He was returned to the intensive care unit and assist-feeding was resumed as needed for inappetence. On April 24, Chip was manually restrained for venipuncture. A CBC revealed a persistent leukocytosis (21,340/ul) characterized by 54% heterophils, 12% lymphocytes, and 24% monocytes. His hematocrit was recorded as 36%, his total protein was recorded as 3.8 g/dl by refractometer, and his uric acid was elevated at 16.3. A cross-table, full-body radiograph was obtained and revealed a progressively poor body condition with no subcutaneous fat stores. The previously described severe alveolar pulmonary pattern had progressed and was affecting nearly the entire right and left lung, and coelomic effusion was noted.

Case Outcome

On April 26, 2019, Chip was humanely euthanized due to clinical progression and poor prognosis. Necropsy was performed following euthanasia. At necropsy, Chip had an extremely

poor body condition score of 1.5/9. Widespread muscle atrophy was noted, and his mucous membranes were pale. Approximately 10 milliliters of thin, clear to pale yellow coelomic effusion was present in the coelom. All air sacs were apparently clear of gross lesions, and the trachea exhibited several yellow to green irregularly shaped and sharply demarcated plaques tightly adhered to the tracheal mucosa and surrounded by a zone of hyperemia. The left lung contained a large green, fibrous, firm mass measuring approximately 3 cm x 1.5 cm x 8 mm. Discoloration was noted throughout the mass, and all pulmonary structure was apparently destroyed due to massive infiltration. The right lung contained similar green to yellow infiltrative growth approximately 1 mm thick and poorly organized throughout the pulmonary parenchyma. Several yellow to tan, irregularly round, sharply demarcated masses ranging from approximately 5 mm to 1 cm in diameter were present throughout the superficial parenchyma. The liver was mildly enlarged with rounded caudal margins. A single tan to yellow, irregular plaque was noted on the left lobe of the liver, and the left kidney was apparently enlarged and pale. Cultures for *Aspergillus* species were not obtained at the time of necropsy; tissues were submitted for histopathology.

Avian aspergillosis can present significant diagnostic and therapeutic challenges as demonstrated in this case report. Unfortunately, aspergillosis is routinely complicated by the presence of comorbidities, which may contribute to the initial immunosuppression of the bird or make the bird poorly responsive to medical management. The development of increasingly reliable antemortem diagnostics and methods of prevention are vital to successful captive management and promotion of this endangered species.

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