Attachment 2 - Abstract

Wildlife Health Center
Oiled Wildlife Care Network

PROJECT ABSTRACT

State the objectives, specific aims and the significance of the project, and describe the methodology used to achieve these goals. Avoid summaries of past accomplishments. The abstract is meant to serve as a succinct and accurate description of the work when separated from other portions of the proposal. Do not exceed the space allowed; 10 pt. font and single-spacing is allowed for this section only. Do not use abbreviations in the title.

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FUNDING AMOUNT REQUESTED:

PROJECT TITLE: THE ECOLOGY OF ASPERGILLOSIS IN SEABIRDS: EVALUATION AND VALIDATION OF AVAILABLE DIAGNOSTIC TESTS

Abstract:

Although *Aspergillus* sp. are ubiquitous fungal organisms in the environment, they (and, in particular, *A. fumigatus*) often has devastating consequences to debilitated avian species. One bird subgroup especially susceptible to infection and subsequent high mortality levels are rehabilitated seabirds – specifically, those recovering from and treated during oil spills. Identified risk factors for developing this disease that are often present in oil spill rehabilitation include "stress" (from capture, housing, and handling), a damp environment with little ventilation, damage to upper airway mucosal epithelium from caustic petroleum products, and concentrated environmental exposures from food, bedding, and conspecifics. Lesions are predominantly restricted to the lungs and air sacs, resulting in a range of clinical signs including dyspnea, voice change, anorexia and/or acute death.

A large number of available diagnostic tests exist to help aid in diagnosis of this disease in other avian and mammalian species. Available "tests" include physical examination (auscultation), blood analyses (complete blood counts and/or serum chemistry), radiology, PCR, and antigen/antibody ELISAs. The latter two classes of tests show promise in diagnosis of this disease in other species, however, there has not yet been a concerted effort towards validating these tests in seabirds at risk during oil spills. Also, in rehabilitation situations, the cost, ease, and value of testing are all important considerations in selecting appropriate diagnostic tests – considerations not necessarily taken into account for other animal species.

It is critical to find a method to detect this disease early in oiled seabird rehabilitation due to the poor prognosis of moderately/severely infected individuals, the potential zoonotic transmission of this disease to oil spill workers, and the difficulty in detecting sub-clinical infections in affected animals. Therefore, this study will strive to test and validate most available diagnostic tools again current antemortem "gold standards" of tracheal wash/culture and necropsy on susceptible seabirds that die or are humanely euthanized during course rehabilitation at the San Francisco Bay Oiled Wildlife Care and Education Center between October 2005 and June 2006. The results of this study will help determine which (if any) available test or tests are the most appropriate to run during an oil spill response, and help to further the OWCN's mandate of providing the "best achievable care" to oil-affected wildlife.

BACKGROUND INFORMATION

Aspergillus sp. are ubiquitous fungal organisms present in the environment, and are responsible for the most common and devastating infectious disease in captive or rehabilitated seabirds: aspergillosis. Transmission usually occurs by inhalation of infective spores, and the disease is thought to be primarily from infection by *A. fumigatus*. Lesions are predominantly restricted to the lungs and air sacs, although a disseminated form can cause systemic illness[1]. Factors suspected to increase the risk of disease include high environmental spore exposure, poor ventilation, inadequate nutrition, environmental stressors, and pre-existing disease prevalence[2]. Other factors that may predispose rehabilitated birds to infection include captive stress, antibiotic or corticosteroid use[3].

Oiled seabirds are especially susceptible to this disease because of the protracted step-wise rehabilitation necessary to recover the damage caused by oil exposure and to re-establish waterproofing of their feathers. Often the rehabilitation facilities used during spills are designed and "built" at the time of the spill event, and use pre-existing structures which often do not have adequate ventilation, heating/air conditioning or biosecurity. Husbandry conditions also provide a perfect environment for fungal growth and transmission in that, due to large numbers of birds cared for, a large amount of organic material (food and feces) often remains in the animal's environment for protracted periods, large numbers of birds are often housed in close proximity to one another, and husbandry techniques used to care for hundreds of birds often do not allow for ideal disinfection protocols to be followed. Release rates for wildlife affected during oil spills have increased due to advances in care and management methods and techniques[4,5], but the detrimental effect of aspergillosis infections remain.

A suspected diagnosis of aspergillosis is frequently made based on clinical impression (attitude and/or results of auscultation), although CBC results (pronounced persistent heterophilia, +/- monocytosis) may provide additional information when performed. Even though aspergillosis affects the respiratory system, reliance on lung auscultation alone for diagnosis can be misleading, as the plaques created by the fungus are not diffuse throughout the pulmonary parenchyma so will often not alter lung sounds. These and other antemortem diagnostic techniques for aspergillosis have been difficult to perform and interpret in rehabilitation settings, especially with conflicting information and inadequate data supporting optimum diagnostic protocols in these birds. Consequently, not only is there is a great need to find ways of minimizing exposure, but also finding practical diagnostic tests that can be used to diagnose infected birds, which will ultimately improve treatment and management.

Currently, there are several tests that have been developed and are commercially available to diagnose aspergillosis in different species, but none have been focused on bird species often affected during oil spills. Multiple ELISAs are available for aspergillosis testing in humans and psittacine and raptor species, but have not yet been validated in seabirds. The two tests created for birds include an *Aspergillus* sp. antibody titer, available through the Raptor Center at the University of Minnesota, and an antibody/antigen test performed at the University of Miami's Avian and Wildlife Laboratory. Published results using these diagnostic tools have been mixed, and many feel the results are unreliable without confirmatory or alternate testing[6,7]. Another ELISA method (focused on antigen capture of galactomannan) has been developed to diagnose invasive aspergillosis in humans[8]. This method, though, requires further investigation in avian species, as the one study focused on avian species found that antigen positive tests did not correlate well with antibody test results and the study used few confirmed positive (by culture or necropsy) animals as a "gold standard"[9]. A polymerase chain reaction (PCR) test that can be used to detect *Aspergillus* sp. in tracheal washes and lung tissue is currently being evaluated in Anseriformes, but it is unclear if this method would be able to be modified for spill use. A Beta-D-glucan assay, developed as an adjunctive diagnostic technique for human invasive fungal infections[10], is also being evaluated as a diagnostic tool in Anseriform species (Tell, unpublished data). Serum protein electrophoresis has previously shown some promise as an adjunct diagnostic tool to determine aspergillosis in birds. Beta and gamma globulins have been shown to increase in infected birds, while low albumin levels have been associated with poorer prognosis in penguins[11]. Other

consistent changes seen on electrophoresis in other avian species include an increased Beta-globulin level, decreased albumin, and a decreased A/G ratio[12]. Other diagnostic tools that have been used to diagnose avian aspergillosis include radiography, tracheal wash with cytology and culture, endoscopy and potentially MRI or CT. Currently, tracheal wash and/or endoscopic sampling plus culture are considered as antemortem "gold standards" even though these techniques are not 100% sensitive. Necropsy, histopath and culture remain the definitive method of diagnosis in animals that succumb to the disease.

Due to the high prevalence of *Aspergillus* sp. infections in seabirds during spills, the poor prognosis of moderately/severely infected individuals, the potential zoonotic transmission of this disease to oil spill workers, and the difficulty in detecting sub-clinical infections in affected animals, it is critical to identify a test (or determine a series of appropriate diagnostic tools) to detect this disease in susceptible species in order to provide the "best achievable care" to oil-affected wildlife. Therefore, the overall goal of this study is to validate available diagnostic tests for aspergillosis in seabirds to determine which (if any) would be most useful as a screening tool during oil spill responses.

HYPOTHESES AND SPECIFIC AIMS

Hypothesis: Available diagnostic procedures and tests developed for aspergillosis in other species, used either singly or in parallel, will be useful for rapid diagnosis in seabirds during oil spill responses.

Aims:

1. Initially screen and prospectively follow seabirds undergoing rehabilitation at the San Francisco Bay Oiled Wildlife Care & Education Center for aspergillosis using available diagnostics.
2. Necropsy, culture, and perform histopathology on study birds that either die or are euthanized during rehabilitation to confirm presence or absence of disease.
3. Statistically determine the optimal combination of diagnostic tests (using pathologic evaluations as the "gold standard") as predictors of disease.

EXPERIMENTAL PLAN

Project Location/Equipment: This project will primarily take place at the San Francisco Bay Oiled Wildlife Care and Education Center (SFBOWCEC), a federal and state licensed rehabilitation facility designed primarily for the rehabilitation of seabirds that routinely receives approximately 3,000 oiled, injured, ill and orphaned aquatic birds each year. This program, jointly managed by the International Bird Rescue Research Center (IBRRC) and the UC Davis - Wildlife Health Center (WHC), has an ongoing program to rescue, rehabilitate and release aquatic birds, and the facilities are equipped with an examination room, an intensive care unit, sample collection equipment, a surgery suite, a laboratory for diagnostic testing, and a necropsy room. An in-house radiograph unit, as well as automatic processor, is already present in this facility. An isoflurane anesthetic machine is also available for use during anesthetic procedures. Housing cages specialized for aquatic birds that avoid injury and provide optimal ventilation exist in all treatment rooms. Outdoor and indoor pools for housing individuals prior to release are compatible with best practices for these species. As avian species are one of the few animals that shed conidiophores (fungal forms potentially infective to humans), appropriate precautions will be taken. Those investigators performing procedures at risk of promoting aerosolization of conidiophores, such as tracheal wash procedures and necropsy, will be fitted for and wear appropriate N95 masks in order to minimize exposure.

Case selection: A sample size of 100 sampled seabirds comprised of the most at-risk families for aspergillosis (Gaviidae, Podicipedidae, and Alcidae) that are presented to SFBOWCEC between Oct. 1, 2005 and June 30, 2006 will ultimately be enrolled in this prospective study. While all birds will initially be enrolled, those successfully released will subsequently be right-censored for the purposes of the validation analyses and only those birds that die on their own or those that are euthanized for humane reasons will be entered in the study. The sample size of 100 was based on expert opinion of disease

prevalence among sick birds, which is estimated between 30 and 50% (M. Ziccardi, pers. comm.) Animals euthanized on intake examination will act as "controls" for this study in that, by not undergoing extended rehabilitation at the SFBOWCEC, it is less likely that they will have *Aspergillus* sp. infections. All birds undergoing rehabilitation will receive prophylactic antifungal therapy (itraconazole, 15 mg/kg PO q24h) as prescribed by the Oiled Wildlife Care Network's "Protocols for the Care of Oil-affected Birds". Additionally, depending on the particular dynamics of oil spill events, birds recovered during active oil spills that occur during this time frame may be included in the study.

Diagnostic test evaluation: Diagnostic tests to be evaluated include physical exam (auscultation), general laboratory diagnostics [complete blood count (CBC), chemistry panel (particularly AST and bile acids) and plasma protein electrophoresis], radiography, tracheal lavage with cytology/culture, serology (ELISA methods), and polymerase chain reaction (PCR) tests (see Table 1 for testing schedule). Upon intake, each study animal will be sampled for diagnostic testing and given a full physical exam. In order to decrease the potential for stress-induced leukocytosis, upon presentation, each bird will be removed from the transport container, a blood sample will be immediately collected for all blood-based tests, and length of handling time prior to sample collection will be quantified. Next, a physical examination will be conducted and the abnormal breath sounds will be rated on a scale of 1-4, ascending with increasing severity. Ratings will be determined at the point of maximum intensity (PMI) which will be characterized based on its regional distribution (e.g., left or right cranio-dorsal quadrant, left or right cranio-ventral quadrant) and descriptive character (e.g., wheeze, crackle). Complete blood counts will be performed by the investigators at the SFBOWCEC using the Unopette eosinophil method.[13] Serum samples will be spun down, separated, then either analyzed immediately (serum chemistry and galactomannan ELISA – J. Burco) or stored at -70^{o} C for shipment (ELISA and electrophoresis - University of Miami Avian and Wildlife Laboratory; ELISA - University of Minnesota Raptor Center; beta-D-glucan detection assay – Associates of Cape Cod, Inc.). After intake and an initial stabilization period of 24 hours, birds will be anesthetized using isoflurane and intubated using a sterile endotracheal tube, radiographed (ventrodorsal and right lateral views) while the respiratory system is inflated using positive pressure ventilation, and resultant radiographs will be read by an avian specialist (L. Tell) for evaluation of lesions consistent with aspergillosis.[14] While under anesthesia, a tracheal lavage will be performed and samples submitted for fungal culture and cytology (UCD - VMTH) and PCR (L. Tell). For serial sampling, radiographs will be performed on a weekly basis for a maximum of 3 evaluations. Tracheal lavage will be performed on intake (after stabilization) and repeated either after one week in captivity or pre-euthanasia. A portion of the lavage sample will be placed in an EDTA tube for cytological examination via a Cytospin technique. These samples will be refrigerated for a maximum of two hours before transfer to UCD-VMTH for analysis. Complete blood counts, plasma chemistries, electrophoresis, and antigen-based tests will be repeated every 3-5 days until a maximum of 4 samples have been collected. Antibody-based tests will be performed upon intake and repeated 10-14 days later to allow sufficient time for an appropriate immunoglobulin response.[15] Antigen tests such as PCR and ELISA will also be run on the same schedule.

Table 1: Schedule of Diagnostic Testing for *Aspergillus* sp.

Diagnostic Test	What Day During Rehabilitation Test Will Be Conducted
Physical Exam	0
CBC/ Chemistry Panel	0, 3-5, 6-8, 9-12
ELISAs/Beta-d-Glucan	0, 10-14
Radiographs	1, 8, 15
Tracheal Lavage & Culture/Cytology	1, 8
PCR	0, 10-14

Note: Samples will be collected for analysis prior to euthanasia if performed

Pathological evaluation: For birds that either die or are humanely euthanized, full gross and histopathological evaluations will be performed by, or under the direct supervision of, a board-certified pathologist (J. St. Leger) to determine the presence or absence of *Aspergillus* sp. lesions/organisms. The respiratory system (air sacs, trachea, and lungs) and visceral organs, in particular, will be targeted for scrutiny. Samples for culture will immediately be collected and sent to UCD-VMTH for analysis. As part of another study to be performed and funded by the Co-PIs, those samples that are culture positive will then have itraconazole HPLC sensitivities performed in order to determine if therapeutic levels of itraconazole are being achieved.

Data analysis: Sensitivity and specificity of the various tests for use in seabirds will be estimated based on the confirmed diagnosis from pathology and/or culture and using general epidemiological methods. Multiple logistic regression (using pathological diagnosis and/or culture results from tracheal wash as the outcome) will be performed in order to determine which diagnostic test(s) best predict(s) the presence of aspergillosis infections, as well as the best model for prediction of clinical disease. All analysis will use appropriate statistical software (Epi Info, CDC, Atlanta, GA USA; SPSS, Chicago, IL USA) and p values less than 0.05 will be considered significant for all analyses.

SIGNIFICANCE TO OILED WILDLIFE HEALTH

Although *Aspergillus* is a ubiquitous fungal organism in the environment, it often has devastating consequences to debilitated avian species. One of the avian subgroups especially susceptible to infection and subsequent high mortality are rehabilitated seabirds, especially those recovered from oil spills. A thorough analysis of other direct systemic effects of petrochemicals on specific organs and blood parameters has been performed by Leighton[15]. Potential for secondary immunosupression and development of secondary complications or opportunistic infections such as aspergillosis have also been identified. Risk factors for developing this disease that are often seen in oil spill response and rehabilitation scenarios include “stress” (from capture, housing, and handling), a damp environment with little ventilation, damage to upper airway mucosal epithelium from caustic petroleum products, and concentrated environmental exposures from food and bedding, and conspecifics. Clinical signs of avian aspergillosis vary, but may include dyspnea, voice change, anorexia or acute death. A large number of available diagnostic tests exist to help aid in diagnosis of the disease in other species, but there has not yet been a concerted effort towards validating these tests in seabirds. In rehabilitation situations, the cost, ease, and value of testing are all important considerations in selecting appropriate diagnostic tests. Some studies have examined the value of currently available avian serology tests for *Aspergillus* detection, but they have not been well explored in the species commonly encountered during oil spill response[13, 16]. The recent development of several antigen-detection diagnostic tests show promise, as they are more specific in detecting *Aspergillus* sp., while combined antigen and antibody tests have been shown to be useful in the detection of disease as well as response to treatment.[17] Overall, this study will be useful in helping dictate which diagnostic tests would be the most appropriate to run in a spill response setting in order to detect disease early enough for treatment to be effective, and for those released animals to have the best chance at long term survival.

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