DETECTION OF GLIOTOXIN IN PLASMA FROM BIRDS WITH CONFIRMED AND PROBABLE ASPERGILLOSIS

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Running Head: Gliotoxin Detection in Avian Aspergillosis

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Abstract: Aspergillosis remains a difficult disease to diagnose *antemortem* in many species, especially avian species. In the present study, banked plasma samples from various avian species were examined for gliotoxin (GT), which is a recognized key virulence factor produced during the replication of *Aspergillus sp.* hyphae, and a secondary metabolite bis(methyl)gliotoxin (bmGT). Initially, the validation of liquid chromatography-tandem mass spectrometry methods for detecting GT and bmGT were conducted in a controlled model using sera obtained from rats experimentally infected with *Aspergillus fumigatus*. The minimum detection level for both measurands was determined to be 3 ng/mL and the assay was found to be accurate and reliable. As proof of concept, GT was detected in 85.7% (30/35) of the samples obtained from birds with confirmed aspergillosis and in 60.7% (17/28) of samples from birds with probable infection but only in 1 of those from clinically normal birds (1/119). None of the birds were positive for bmGT. Positive repeated measures from birds under treatment suggests results may have prognostic value. Further studies are needed to implement quantitative methods and to determine the utility of this test in surveillance screening in addition to its use as a diagnostic test in birds with suspected aspergillosis.

INTRODUCTION

Invasive infection by *Aspergillus* species, primarily *Aspergillus fumigatus,* remains a major cause of morbidity and mortality in immunocompromised humans, especially after bone marrow transplantation or cancer chemotherapy.[2] In veterinary medicine, aspergillosis is responsible for high mortality across all avian species.[8] Similar to human medicine, *antemortem* diagnostic options in avian species are limited and include routine hematology, assessment of inflammation, serological testing, culture, and imaging.[13,29] As many avian species show anti-fungal antibody reactivity in the absence of infection, the appearance of an acute phase response using plasma protein electrophoresis in tandem with clinical signalment, and imaging, is often used to aid in diagnosis.[9-11,16]

Over the last several years, many investigators have sought to study and implement novel test options, which have led to the development of antigen-based testing for galactomannan and beta-D-glucan for application in both human and veterinary medicine.[2,4,9] So far, these biomarkers were not shown to be reliable for the diagnosis of aspergillosis in birds. Alternatively, other researchers have proposed that metabolite-based diagnostics, including gliotoxin (GT) detection, may be viable markers of aspergillosis.[5,22,30] GT is the most abundant mycotoxin produced by clinical isolates of *A. fumigatus*, although it can also be found in other *Aspergillus* species as well as other fungal genera considered non-pathogens such as *Penicillium*, *Trichoderma*, and *Leptosphaera*.[20,23,25] It has been identified as a key virulence factor that results in far-reaching immune suppression.[1,21] Studies have demonstrated that GT inhibits phagocytosis, blocks inflammatory processes and cytokine production, and induces apoptosis in macrophages and monocytes.[21] Using *in vivo* models, survival time of immune suppressed mice was extended when *A. fumigatus* was used where gliotoxin was genetically deleted.[21] Several

studies utilizing samples obtained from mammal models and banked human sera have indicated that GT detection has a strong potential as a diagnostic test for aspergillosis.[5,22,30] In addition to GT, a secondary metabolite bis(methyl)gliotoxin (bmGT) has also been detected and was reported to have better sensitivity than GT.[17,32,33]

In the present study, the technical parameters for detecting GT and bmGT in avian plasma through high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) methodology have been presented. Although GT has already been measured in tissues from turkeys and chickens naturally infected with *A. fumigatus*,[12,27] this is the first report of GT detection in the plasma of various avian species with confirmed and probable aspergillosis.

MATERIALS AND METHODS

Ethics

The rat study was reviewed and approved by the Institutional Animal Care and Use Committee at Tours University (France) as No. C37-261-3 and by the General Direction for Research and Innovation, French Ministry of Higher Education and Research through the accreditation number 01901.01. For the avian study, samples were submitted either as part of a routine or planned diagnostic analyses to the Avian & Wildlife Laboratory (University of Miami, Miami, FL, United States) or as part of SSP study protocol requesting banked samples from different AZA participating institutions. As part of the latter process, consent was received from participating institutions. No institutional approval (at the University of Miami) was required.

Rat Model

Male Sprague-Dawley rats (n=20), Janvier Labs, Le Genest-Saint-Isle, France), 6-8 weeks old, 200-225 g, were acclimated in animal facilities eight days before the beginning of the protocol. On day 0,all animals were immunocompromised intraperitoneally with 75 mg/kg cyclophosphamide (Baxter, Guyancourt, France), and their food was changed to a low-protein diet (Safe Diets, Augy, France). To avoid opportunistic infection and limit pain, 500mg/L tetracycline (Sigma-Aldrich, Saint Quentin Fallavier, France) and 300 mg/L paracetamol (Sanofi-Aventis, Montrouge, France) were added to their drinking water. A second cyclophosphamide administration of 60 mg/kg was given on days 4, 8 and 12 to maintain immunosuppression. According to the previously published protocol,[6,14] animals were challenged intratracheally at day 5 by aerosolization of 100 µL of a PBS suspension containing $1.0x10^6$ *A. fumigatus* conidia with Microsprayer IA-1B® aerosolizer (n=11, PennCentury, Philadelphia, PA, USA) or with PBS alone as a mock-infected control group (n=9). Rats were then monitored daily and euthanasia was performed before the end of the protocol if the following criteria were met: loss of weight ≥20%, discomfort score 3 (scored from 1 to 6 based on appearance changes, *e.g.*, dirty nose, red-rimmed eyes, ruffled fur, extreme pallor; score 1, no discomfort; score 2, minor discomfort; score 3, poor discomfort; score 4, serious discomfort; score 5, severe discomfort; score 6, death), behavior changes (*e.g.*, gasping, wheezing, prostration, instability) and decreased reaction to stimuli. These criteria were met between day 8 and 15. At necropsy, blood and lungs were collected for subsequent analysis. To verify experimental aspergillosis development, qPCR, targeting the *A. fumigatus* 28S ribosomal DNA 28S, was carried out in lung tissues as previously described.[7] Serum was analyzed for galactomannan presence using the Platelia Ag kit (Bio-Rad, Marnes-la-Coquette, France) and also banked at -80°C for analysis of GT and bmGT.

Avian Samples

Heparinized plasma samples were obtained from routine health surveillance programs, and diagnostic investigations from birds with suspected aspergillosis were analyzed in addition to samples obtained as part of the AZA SSP program (see Acknowledgements). In some cases, repeated measures were available. Many of the samples had been stored at -80^{o}C for extended periods. The samples (n=119) from clinically normal penguins (n=51 individual birds) were obtained from a single facility: they were in normal condition based on physical examination and bloodwork and did not show any clinical evidence of aspergillosis. Twenty-five of these samples were obtained from penguins that did not show any clinical evidence of aspergillosis at least 6 months after sampling. Ninety-four of the samples were taken over a recent 3-month period and the penguins also remained clinically normal during this period. Two clinically abnormal groups were defined and included confirmed – those with histopathological confirmation after necropsy or consistent radiography and culture results – and probable – those with consistent clinical signs, supporting testing including radiography, and a positive response to anti-fungal treatment. Species represented in the confirmed sample group (n=35) ranged from 2 months to 13 years of age and included the African penguin (n=8, *Spheniscus demersus*), Humboldt penguin (n=6, *Spheniscus humboldti*), gentoo penguin (n=4, *Pygoscelis papua*), California condor (n=4, *Gymnogyps californianus*), whooping crane (n = 1, *Grus americana*), little blue penguin (n=2, *Eudyptula minor*), grey-winged trumpeter (n=2, *Psophia crepitans*), Magellanic penguin (n=3, *Spheniscus magellanicus*), helmeted curassow (n=4, *Pauxi pauxi*), and wreathed hornbill (n=1, *Rhyticeros undulatus*). Note that 17/35 samples represented repeated measures over several months in 5 cases. Those in the probable sample group (n=28) ranged from 8 months to 13 years of age and included the African penguin (n=14), Humboldt penguin (n=9), little blue penguin

(n=1), Magellanic penguin (n=1), gyrfalcon (n=1, *Falco rusticolus*), pigeon (n=1, *Columba livia domestica*), and African grey parrot (n = 1, *Psittacus erithacus*). Note that, overall, 7/28 samples represented repeated measures over several weeks to months in 3 cases.

Chemicals and Materials

All solvents and reagents were purchased at HPLC grade unless noted. Ammonium acetate, ammonium formate, ammonium hydroxide, American Chemical Society (ACS) grade formic acid, glacial acetic acid, ACS grade diethyl ether, ACS grade ethyl acetate, LC-MS grade water, LC-MS grade acetonitrile, and methanol were purchased from VWR (Randor, PA, United States). Certified human negative serum was purchased from Bio-Rad (Hercules, CA, United States). Drug standards, which included gliotoxin and bis(methylation) gliotoxin, were purchased from Millipore Sigma (Burlington, MA, United States). Internal standards (IS), including mycophenolic acid-D4 and TFMPP-D3, were purchased from Cerilliant Corporation (Round Rock, TX, United States).

Sample Preparation

All stock solutions, negative controls, and specimens were allowed to equilibrate to room temperature for at least 30 minutes. After equilibration, a 100 µL aliquot of the specimen was fortified with 10 µL of the working internal standard solution for a final internal standard concentration of 100 ng/mL. Next, 1.2 mL of diethyl ether: ethyl acetate (50:50 *v/v*) was added to each tube and vortexed for approximately 30 seconds. Specimens were centrifuged at 2851 g (RCF max) for 10 min, and once complete, the supernatant was transferred to a clean tube. The eluent was then evaporated at 25°C with N_2 and reconstituted with 50 µL of the 50:50 A: B (*v/v*)

mobile phase composition. Mycophenolic acid-D3 and TFMPP-D4 were used as internal standards.

Instrumental Analysis

An Agilent Technologies 1260 liquid chromatogram coupled to a 6460 triple quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA, United States) equipped with a Waters XBridge BEH Shield RP18 analytical column (130Å, 5 µm, 2.1x150mm) and Waters XBridge BEH C18 Sentry guard cartridge (130Å, 5 µm, 2.1 x 10mm) (Waters Corporation, Milford, MA, United States) was utilized for chromatographic separation and identification of the gliotoxin and its metabolite. The analytical column was maintained at 40°C in the temperature-controlled column compartment. The mobile phase consisted of 5 mM ammonium formate with 0.1 % formic acid in LC-MS grade water (A) and LC-MS grade acetonitrile with 0.1% formic acid (B). The mobile phase gradient was programmed as follows: 95% A was held for 1 minute, 10 min, then decreased to 10% A over 9 minutes, with a return to 95% A at t=11 min. A 2-min post-run time was used to re-equilibrate the column before the subsequent injection. The injection volume was set at 5 µL.

Mass spectral data were acquired using an Agilent Jet Stream electrospray ionization (ESI) source operated in positive ion mode. The ESI source and MS parameters are as follows: gas temp of 320°C, flow 8 L/min, nebulizer 27 psi, sheath gas heater 380°C and flow 12 L/min, the capillary voltage at 3,750 V, and the nozzle voltage 500. The instrument was operated in dynamic multiple reaction monitoring (dMRM) acquisition mode with the unit resolution was utilized for all analytes and transitions. Initially, the Agilent Optimizer software was used to identify product ions and their optimal fragmentor voltage and collision energy to determine

which transitions should be used for each target analyte and internal standard. Two transitions were monitored for each analyte and internal standard. MRM transitions are shown for each analyte and internal standard in Table 1.

Each analyte minimum identification criteria included: retention time within ±3% of the positive controls, a Gaussian chromatographic, peak shape, signal-to-noise greater than 3:1, and qualifier transition ratios within acceptable ranges. Figure 1 depicts a chromatogram with gliotoxin, retention time 6.7, and the IS, Retention Time 5.6 mins with the specified transitions.

Method validation parameters

Limit of Detection

The approximate LOD of the analytes was determined using a 0.5 ng/µL stock solution to fortify negative penguin sera at the following concentrations: 1, 3, 5, 10, and 15 ng/mL. Fortified specimens were prepared using six different negative penguin sera specimens over three runs (n=12). The analytes had to meet the previously stated retention time, peak shape, and qualifier ion ratio criteria in all 12 replicates to be considered detected. Additionally, the signal-to-noise for both transitions had to be greater than 3:1 as calculated by the Agilent MassHunter® software using the General integrator setting.

Linearity

Calibrators were prepared in certified negative human serum to span the calibration range, analyzed over five separate batches, and statistically analyzed to establish the calibration range. In addition, multi-level positive controls prepared in species-matched specimens were

analyzed alongside the calibration curve to verify accuracy in a specified matrix and the bias and precision of the analytical method.

Interferences Studies

To ensure that the target analyte itself only produces a positive identification for the target analyte, interference studies were performed. In each experiment, interference was determined to be present if a signal met the identification criteria, which included: retention time, signal-to-noise greater than 3:1, peak shape, and qualifier ion transition ratios.

To investigate if any potential interference from endogenous compounds in the matrix, six (6) sources of rat negative matrices and fifteen (15) sources of penguins were analyzed without the addition of the IS. All negative samples were pre-screened and had no history of illness or infection with *Aspergillus fumigatus*.

The stability and purity of the deuterated internal standards were also assessed. To verify the deuterated internal standards' purity, three different negative sera specimens were fortified with the internal standard solution at the same concentration as sample analysis to determine if any interference was observed.

Ionization Suppression/Enhancement

Suppression or enhancement of the analyte signal can occur when using LC-electrospray mass spectrometry (LC-ESI-MS). This can be caused by co-eluting compounds that can originate from the matrix itself or the extraction. Ion suppression or enhancement typically affects method parameters such as the limit of detection in qualitative assays.

To assess ionization suppression and enhancement, the average peak areas from 6 replicates of neat standards (set A) were compared with the average peak areas from ten fortified

specimens (set B). The following formula was used to assess ionization suppression and enhancement:

$$\text{Ionization suppression or enhancement (\%)} = \left(\frac{\bar{X}\ \text{Peak Area of Set B}}{\bar{X}\ \text{Peak Area of Set A}} \right) \text{x } 100\% \quad \text{Eq 1.}$$

Recovery was also investigated using pre- and post-fortified samples from different penguin sera.

RESULTS

Method Validation

Limit of Detection (LOD) and linearity

Analyzing the results of the LOD study, GT and bmGT were established to have a LOD at 3 ng/mL. The appropriate calibration model was determined through statistical analysis using standardized residual plots and ANOVA to be a linear model with a weighting factor of 1/x for concentrations spanning 10-1000 ng/mL for GT and bmGT. The limit of detection (LOD) was determined as 3 ng/mL by evaluating peak shape and using the standard signal to noise (S/N) ratio of 10 and acceptable mass spectrometric criteria. This was established by utilizing six sources of rat negative matrices and fifteen sources of penguin negative matrices that were fortified with GT and bmGT. The analytical method was able to identify both compounds in all matrix specimens. The lower limit of quantitation (LLOQ) was determined through repeated measurements at 10 ng/ml for both compounds in three different matrices, duplicate, and three different batches (n=9). Chromatographic and mass spectrometric interference was not observed in the endogenous, exogenous, and internal standard studies, and no carryover was detected with

a serum fortified at 500 ng/ml into the subsequent sample. There was, however, significant variation in the signal of the internal standard observed in the different penguin samples indicative of possible matrix effects and/or differences in extraction efficiencies. The observed variation appeared to affect the accuracy and precision of the calculated concentrations; therefore, it was determined that only qualitative results would be reported using this method with this internal standard.

Ionization Suppression/Enhancement (ISE) and recovery

In qualitative assays, ionization suppression or enhancement can significantly affect the LOD for an analyte. Suppression and enhancement studies using fortified penguin matrices showed an acceptable % ISE of less than 20, and recovery of GT and bmGT were established as 90%.

Rat Samples

Nine samples from the mock-infected control rats were analyzed. All had a serum galactomannan index of <0.14 (where 0.5 or greater is considered positive). Ribosomal *Aspergillus* DNA levels were considered insignificant. All serum samples were found to contain no interference for GT or bmGT.

Eleven infected rat samples were analyzed. In the serum, the galactomannan index ranged from 3.2 to 8.0. Infection was further confirmed by quantitation of ribosomal *Aspergillus* DNA levels obtained from the lung; these ranged from 4092 to 176,583 fg/µL. Serum samples from all 11 infected rats had detectable levels of GT but not bmGT.

Avian Samples

One hundred and nineteen samples were obtained from clinically normal penguins were analyzed. bmGT was not detected in any samples. GT was detected in one sample. While this penguin showed no clinical signs, during this assessment period, the electrophoresis showed abnormalities including increased globulins and there was also evidence of seroconversion by ELISA. In the absence of any treatment, the GT was not detected in any follow up samples, and the electrophoretogram returned to normal.

In birds with probable infection, 17 of 28 or 60.7% of samples had detectable GT and, in birds with confirmed infection, 30 of 35 or 85.7% of samples had detected GT (Table 2). bmGT was not detected in any of the samples.

Repeated measures were available for some of cases examined during the study (Table 3). In cases of confirmed infection, GT was consistently detected in the samples available for analysis. In the single confirmed case that was successfully treated (case 5), GT was detected on all repeated measures obtained when clinical signs were present and in one sample when the bird appeared clinically normal. In a probable case of infection (case 2), GT was not detected after 3 weeks of treatment; systemic inflammation was supported by the presence of an abnormal electrophoretogram, but clinical signs were no longer observed.

DISCUSSION

Serodiagnostic tests for avian aspergillosis are broadly limited to the same options found in human medicine.[29] Antibody testing is problematic as many species of birds have been shown to have high reactivity on an ELISA based on a crude antigen preparation even when clinically normal.[10] Detection methods of circulating antigen, including galactomannan[9] and beta-D-glucan,[4] have been validated but have limited sensitivity for diagnosis. Currently used methods

include detecting an ongoing acute-phase response *via* plasma protein electrophoresis.[9,16] In a search for a more definitive marker of infection in humans, many investigators have focused on the detection of metabolites of fungal infections,[28] including GT, which has been identified as a major virulence factor of *A. fumigatus*, and its metabolite, bmGT.[1,5,17,22,30,32,33]

In the current investigation, preliminary studies were undertaken to validate an HPLC-MS/MS method using serum samples from an acute rat model of invasive aspergillosis. This model was selected as it has been well characterized and reproducible.[6,15] This data corroborated previous reports using mouse models where GT was readily detected, which validated the implementation methodology.[22,30] Notably, in the present study, GT was not detected in samples from mock-infected control rats thus supportive of excellent assay specificity.

Importantly, GT was not readily detected in a large cohort of clinically normal penguins, an avian species which has been shown to have a high prevalence of aspergillosis when in captive care.[3,8,19] Although limited in sample size, the current preliminary study has detected GT in cases with confirmed or probable infection. These results are consistent with those previously reported in studies utilizing human and rat samples.[5,22,30] While the present study did not rule out infections with other fungal species, it should be noted that GT can also be produced by genera that are generally regarded as non-pathogens.[25]

The sensitivity for detecting avian aspergillosis by GT has been difficult to assess, although the detection rate was higher in confirmed rather than probable cases. It has been proposed that GT would be a sensitive marker of early infection as it is produced during hyphal replication.[34] Analysis of archival plasma samples pre-, during, and post-diagnosis presents

challenges due to variability in the timing of collected samples that are available. Additionally, definitive diagnosis *via* culture or molecular diagnostics is difficult *antemortem*, particularly in chronic cases, and may only be confirmed by necropsy where often only histopathological analysis is completed. Cross-sectional imaging (computed tomography) and positive response to treatment are often used clinically to guide treatment and monitor advanced disease cases without a definitive diagnosis. Classification of advanced or chronic cases can be problematic as these birds may have a less severe disease (thus a positive outcome) or are either not suffering from aspergillosis or are infected with a non- or low gliotoxin-producing isolate of *Aspergillus*. Thus, the scope of the present study should be considered preliminary. First, although the samples considered to be control samples (n=119) were obtained from penguins that remained in good health post sampling, there were no detailed investigations to rule out the presence of colonization with *Aspergillus* or aspergillosis. Second, confirmed cases were limited by histopathology reports only that were obtained from various laboratories, often in the absence of culture, special staining, and molecular confirmation. Lastly, as in many retrospective studies, the use of archived samples with variable storage methods is often suspect although, notably, stability studies of GT conducted in human sera indicated that refrigeration, freeze-thaw, and month-long freezing did not affect results.[5]

Ongoing collaborations with zoological institutions and aquaria are focused on advantageous screening of apparently clinically normal penguins to gauge whether this assay may aid in routine screening protocols. It is important to examine necropsy results in future studies where aspergillosis has been confirmed in the absence of gliotoxin detection. Also, while *A. fumigatus* is frequently observed to be the pathogen in avian aspergillosis cases; current study collaborators are urged to perform cultures and/or molecular diagnostics at necropsy with the

goal of identifying the species of fungus. Previously, others have reported GT was detected in most *A. fumigatus* isolates but to a moderate level in *A. niger* and a lower number of isolates of *A. terreus* and *A. flavus.*[20,23] While possible false-negative results may be related to the timing of sampling *vs.* the course of the disease, it is recognized that many isolates of non-*A. fumigatus* species produced lower GT levels, and some clinical isolates of *A. fumigatus* in probable cases did not produce GT.[20] In addition, it has been proposed that penguins are colonized by *Aspergillus sp*. So, it may be argued that the presence of GT may not necessarily reflect clinical infection.[26] It could also be proposed that GT would only be detected in cases where clinical signs are evident and fungal replication is active. GT levels may also decrease with the presence of advanced disease stage where hyphal expansion is slowed.[32] In the present study of clinically normal African penguins, only 1 of 119 samples had detectable GT. While this penguin showed no clinical signs, other tests supported the presence of inflammation and activation of humoral immunity which appeared to resolve in follow up samples. Additional studies with focus on health assessments of clinically normal birds should be conducted to best assess the clinical applications of GT testing.

GT was detected in non-penguin species also. This supports the hypothesis that GT may be used in all avian species, but additional studies should be undertaken to validate these methods in a wider range of species. Repeated measures were available for some of the probable and confirmed cases. In 3 confirmed cases that resulted in death, GT was consistently detected. In one confirmed case with apparent successful treatment, GT was detected at a time with improved clinical signs, so treatment was continued. Although more such cases need to be examined, this suggests that reassessment by GT testing may provide additional prognostic information to the clinician.

Other investigators demonstrated a GT metabolite, bis(methylthio)gliotoxin (bmGT), in human samples. It has been proposed that GT may become incorporated in tissues given its biological activities in apoptosis and cellular damage and that bmGT, which is non-toxic, would be a preferred marker.[17] However, it should be noted that bmGT was not commonly detected in non-*A. fumigatus* isolates.[33] In human patients, the combination of GT and bmGT resulted in excellent predictive value,[32] although others reported bmGT and GT to be unreliable markers.[24] While these differences may be related to the implementation of the LC-MS methods, it may also be a function of the variation in the stages of infection within the cohort of human samples examined in the studies. In the present study, bmGT was not detected in rat or avian blood samples. The lack of detection in rats is consistent with that observed in a mouse model where bmGT was detected in the lungs but not the sera from samples obtained from mouse models of aspergillosis.[30] It was proposed that this may reflect a difference related to using an acute rodent model rather than a more prolonged natural infection often observed in humans.[30] It could be argued that this same proposal should support the detection of bmGT in naturally infected birds where chronic infection is often observed, but it was not the case herein. The lack of detection in rats and avian species may reflect differences in biological processing of metabolites *versus* humans.[30] Additional studies should be undertaken with repeated measures from infected birds or using a model of aspergillosis in a bird species to best assess the application of bmGT detection.

In the present study, penguin plasma matrices were variable which did not allow for accurate GT quantitation and may have also affected bmGT detection. This was also observed in samples from other avian species. Alternative internal standards were investigated but all showed similar variability with these matrices (data not shown). Penguins and other birds with

aspergillosis have been shown to have a significant inflammatory response in conjunction with a decrease in albumin[16]; perhaps this altered plasma composition contributes to the issue of inaccurate quantitation and lack of bmGT detection. Although all criteria were acceptable in order to provide qualitative GT test results with the established 3 ng/mL LOD across multiple sources of penguin and rat matrix, there is a need for the production of a deuterated GT standard to be able to compensate for the differences observed in the penguin matrix. As decreasing GT levels during treatment may prove to be a useful prognostic marker, this is a focus of continuing studies. Extended clinical use of GT testing in birds may be limited by the cost of analysis, sample volume requirements, and turnaround time. A recent publication reported developing a fluorescent-based assay with a superior minimum detection level, which may help make GT detection more readily available for clinical applications in human and veterinary medicine.[18]

The data from this preliminary investigation support the further study of the application of *Aspergillus* metabolite detection in avian species. While this may lead to the development of a more definitive *antemortem* diagnostic test for birds inclusive of many endangered species maintained under managed care, it may also promote the bird as a novel model of aspergillosis,[31] which could provide a further understanding of the pathogenesis of this disease.

ACKNOWLEDGMENTS: The authors thank the African penguin Species Survival Plan (SSP) as part of the Association of Zoos & Aquariums Saving, Animals from Extinction (SAFE) conservation initiative. We also thank all the institutions that shared samples for this study including the Fort Worth Zoo, the Dallas Zoo, and the Freeport-McMoRan Audubon Species Survival Center.

LITERATURE CITED

1. Arias M, Santiago L, Vidal-Garcia M, Redrado S, Lanuza P, Comas L, Domingo MP, Rezusta A, Galvez EM. Preparations for Invasion: Modulation of Host Lung Immunity During Pulmonary Aspergillosis by Gliotoxin and Other Fungal Secondary Metabolites. Front Immunol. 2018;9:2549.

2. Bassetti M, Giacobbe DR, Grecchi C, Rebuffi C, Zuccaro V, Scudeller L, investigators F. Performance of existing definitions and tests for the diagnosis of invasive aspergillosis in critically ill, adult patients: A systematic review with qualitative evidence synthesis. J Infect. 2020;81(1):131-146.

3. Beernaert LA, Pasmans F, Van Waeyenberghe L, Haesebrouck F, Martel A. Aspergillus infections in birds: a review. Avian Pathol. 2010;39(5):325-331.

4. Burco JD, Ziccardi MH, Clemons KV, Tell LA. Evaluation of plasma (1-->3) beta-D-glucan concentrations in birds naturally and experimentally infected with aspergillus fumigatus. Avian Diseases. 2012;56(1):183-191.

5. Cerqueira LB, de Francisco TM, Gasparetto JC, Campos FR, Pontarolo R. Development and validation of an HPLC-MS/MS method for the early diagnosis of aspergillosis. PLoS One. 2014;9(4):e92851.

6. Chandenier J, Bernard S, Montharu J, Bailly E, Fetissof F, de Monte M, Desoubeaux G, Diot P, Richard-Lenoble D. The utility of a nebulised intra-tracheal rat model of invasive pulmonary aspergillosis. Mycoses. 2009;52(3):239-245.

7. Chauvin D, Hust M, Schütte M, Chesnay A, Parent C, Moreira G, Arroyo J, Sanz AB, Pugnière M, Martineau P, Chandenier J, Heuzé-Vourc'h N, Desoubeaux G. Targeting Aspergillus fumigatus Crf Transglycosylases With Neutralizing Antibody Is Relevant but Not Sufficient to Erase Fungal Burden in a Neutropenic Rat Model. Front Microbiol. 2019;10:600.

8. Converse KA. Aspergillosis. In: Thomas NJ, Hunter BD, Atkinson CA, (eds.). Infectious diseases of wild birds. 1st ed. Hoboken, NJ: Blackwell Publishing; 2007. p. 360-374.

9. Cray C, Reavill DR, Romagnano A, Van Sant F, Champagne D, Stevenson R, Rolfe V, Griffin C, Clubb S. Galactomannan assay and protein electrophoresis findings in psittacine birds with aspergillosis. Journal of Avian Medicine and Surgery. 2009;23(2):125-135.

10. Cray C, Watson T, Arheart KL. Serosurvey and diagnostic application of antibody titers to Aspergillus in avian species. Avian Diseases. 2009;53:491-494.

11. Cray C, Watson T, Rodriguez M, Arheart KL. Application of galactomannan analysis and protein electrophoresis in the diagnosis of aspergillosis in avian species. Journal of Zoo and Wildlife Medicine. 2009;40(1):64-70.

12. de Oca VM, Valdes SE, Segundo C, Gomez GG, Ramirez J, Cervantes RA. Aspergillosis, a Natural Infection in Poultry: Mycological and Molecular Characterization and Determination of Gliotoxin in Aspergillus fumigatus Isolates. Avian Dis. 2017;61(1):77-82.

13. Desoubeaux G, Bailly E, Chandenier J. Diagnosis of invasive pulmonary aspergillosis: updates and recommendations. Med Mal Infect. 2014;44(3):89-101.

14. Desoubeaux G, Chandenier J. A nebulized intra-tracheal rat model of invasive pulmonary aspergillosis. Methods Mol Biol. 2012;845:511-518.

15. Desoubeaux G, Jourdan ML, Valera L, Jardin B, Hem S, Caille A, Cormier B, Marchand-Adam S, Bailly E, Diot P, Chandenier J. Proteomic demonstration of the recurrent presence of inter-alpha-inhibitor H4 heavy-chain during aspergillosis induced in an animal model. Int J Med Microbiol. 2014;304(3-4):327-338.

16. Desoubeaux G, Rodriguez M, Bronson E, Sirpenski G, Cray C. Application of 3-hydroxybutyrate measurement and plasma protein electrophoresis in the diagnosis of aspergillosis in African penguins (*Spheniscus demersus*). J Zoo Wildl Med. 2018;49(3):696-703.

17. Domingo MP, Colmenarejo C, Martinez-Lostao L, Mullbacher A, Jarne C, Revillo MJ, Delgado P, Roc L, Meis JF, Rezusta A, Pardo J, Galvez EM. Bis(methyl)gliotoxin proves to be a more stable and reliable marker for invasive aspergillosis than gliotoxin and suitable for use in diagnosis. Diagn Microbiol Infect Dis. 2012;73(1):57-64.

18. Gao S, Zheng X, Tang Y, Cheng Y, Hu X, Wu J. Development of a Fluorescently Labeled Aptamer Structure-Switching Assay for Sensitive and Rapid Detection of Gliotoxin. Anal Chem. 2019;91(2):1610-1618.

19. German AC, Shankland GS, Edwards J, Flach EJ. Development of an indirect ELISA for the detection of serum antibodies to Aspergillus fumigatus in captive penguins. The Veterinary record. 2002;150(16):513-518.

20. Kupfahl C, Michalka A, Lass-Florl C, Fischer G, Haase G, Ruppert T, Geginat G, Hof H. Gliotoxin production by clinical and environmental Aspergillus fumigatus strains. Int J Med Microbiol. 2008;298(3-4):319-327.

21. Kwon-Chung KJ, Sugui JA. What do we know about the role of gliotoxin in the pathobiology of Aspergillus fumigatus? Med Mycol. 2009;47 Suppl 1:S97-103.

22. Lewis RE, Wiederhold NP, Chi J, Han XY, Komanduri KV, Kontoyiannis DP, Prince RA. Detection of gliotoxin in experimental and human aspergillosis. Infect Immun. 2005;73(1):635-637.

23. Lewis RE, Wiederhold NP, Lionakis MS, Prince RA, Kontoyiannis DP. Frequency and species distribution of gliotoxin-producing Aspergillus isolates recovered from patients at a tertiary-care cancer center. J Clin Microbiol. 2005;43(12):6120-6122.

24. Mercier T, Resendiz Sharpe A, Waumans D, Desmet K, Lagrou K, Maertens J. Gliotoxin and bis(methylthio)gliotoxin are not reliable as biomarkers of invasive aspergillosis. Mycoses. 2019;62(10):945-948.

25. Patron NJ, Waller RF, Cozijnsen AJ, Straney DC, Gardiner DM, Nierman WC, Howlett BJ. Origin and distribution of epipolythiodioxopiperazine (ETP) gene clusters in filamentous ascomycetes. BMC Evol Biol. 2007;7:174.

26. Reidardson TH, McBain JF. Diagnosis and treatment of aspergillosis in temperate penguins. Erkrankungen der Zootiere. 1992;34:155-158.

27. Richard JL, Dvorak TJ, Ross PF. Natural occurrence of gliotoxin in turkeys infected with Aspergillus fumigatus, Fresenius. Mycopathologia. 1996;134(3):167-170.

28. Savelieff MG, Pappalardo L. Novel cutting-edge metabolite-based diagnostic tools for aspergillosis. PLoS Pathog. 2017;13(9):e1006486.

29. Savelieff MG, Pappalardo L, Azmanis P. The current status of avian aspergillosis diagnoses: Veterinary practice to novel research avenues. Veterinary clinical pathology. 2018;47(3):342-362.

30. Sugui JA, Rose SR, Nardone G, Swamydas M, Lee CR, Kwon-Chung KJ, Lionakis MS. Host immune status-specific production of gliotoxin and bis-methyl-gliotoxin during invasive aspergillosis in mice. Sci Rep. 2017;7(1):10977.

31. Tell LA. Aspergillosis in mammals and birds: impact on veterinary medicine. Med Mcyol Suppl. 2005;43:571-573.

32. Vidal-Garcia M, Domingo MP, De Rueda B, Roc L, Delgado MP, Revillo MJ, Pardo J, Galvez EM, Rezusta A. Clinical validity of bis(methylthio)gliotoxin for the diagnosis of invasive aspergillosis. Appl Microbiol Biotechnol. 2016;100(5):2327-2334.

33. Vidal-Garcia M, Redrado S, Domingo MP, Marquina P, Colmenarejo C, Meis JF, Rezusta A, Pardo J, Galvez EM. Production of the Invasive Aspergillosis Biomarker Bis(methylthio)gliotoxin Within

the Genus Aspergillus: In Vitro and in Vivo Metabolite Quantification and Genomic Analysis. Front Microbiol. 2018;9:1246.

34. Waring P, Eichner RD, Mullbacher A. The chemistry and biology of the immunomodulating agent gliotoxin and related epipolythiodioxopiperazines. Med Res Rev. 1988;8(4):499-524.

Table 1. MRM transitions for analytes and internal standards

Compound Name	RT (min)	Precursor Ion (m/z)	Fragmentor (V)	Product Ion 1 (m/z)	CE 1 (V)	Product Ion 2 (m/z)	CE 2 (V)
Gliotoxin		327.1	76	263.1	6	56.1	50
bis(methylthio)gliotoxin		357.0	74	309.1	2	243.0	6
TFMPP D4		235.1	125	190	21	46.1	21
Mycophenolic acid D3		341.1	80	210	16		

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Table 2. Summary of Samples Analyzed for the presence of GT.

Case Type	Detected	Not Detected	Percentage Positive
Clinically Normal	1	119	0.8%
Probable Infection	17	28	60.7%
Confirmed Infection	30	35	85.7%

Table 3. Detection of gliotoxin in probable and confirmed cases of aspergillosis with repeated assessments.

Case	Species	Day of Sampling	Gliotoxin Detection	Comments
1	Humboldt penguin (confirmed)	0	Detected	1 mo. weight loss, anorexia, leukocytosis, supportive imaging results, placed on itraconazole as anti-fungal treatment
		7	Detected	No change
		18	Detected	Improved appetite, decreased leukocytosis
		21	Not Done	Minor respiratory difficulty but appetite, attitude, and respirations were normal
		22	Not Done	Animal died, necropsy confirmed chronic granulomatous air sacculitis and multiple pulmonary nodules confirmed with intralesional fungal hyphae
2	Humboldt penguin (probable)	0	Detected	Few-day change in voice noted, leukocytosis, CT scan confirmed diffuse air sacculitis with inspissated contents and pulmonary nodules, placed on itraconazole as anti-fungal treatment
		21	Not detected	Clinically normal although inflammatory pattern of protein electrophoresis continued to be present, continued treatment and follow up imaging normal by day 50
3	California condor (confirmed)	0	Detected	Initial respiratory signs noted (cough), placed on antibiotics
		24	Detected	Weight loss, increase coughing
		28	Not Done	CT scan showed partial collapse of right abdominal air sac, FNA nondiagnostic
		43	Detected	Distended air sacs, increased coughing, death day 45, confirmed infection at necropsy
4	African penguin (confirmed)	0	Detected	Leukocytosis, weight loss, lethargy, weight loss, suggestive imaging, placed on anti-fungal medication
		28	Not detected	Continued clinical signs
		46	Detected	Continued clinical signs, changed anti-fungal meds
		53	Detected	Found dead, confirmed infection at necropsy
5	Helmeted	0	Detected	Respiratory signs, radiographs abnormalities present including likely granuloma,

curassow (confirmed)			cytology revealed fungal hyphae, positive Aspergillus fumigatus culture, paced on anti-fungal medication
	35	Detected	Clinical signs improved but not resolved, medication continued
	66	Detected	Clinical signs improved but not resolved, medication continued
	89	Detected	Clinical signs resolved; medication continued
	117	Not detected	Clinical signs not present, bird released to exhibit with continued medication
	151	Not detected	Clinical signs not present, medication discontinued prior to this timepoint

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Figure 1. Representative chromatogram of gliotoxin and internal standards (IS). The chromatogram shows the elution order and retention times of gliotoxin and the internal standards (IS), TFMPP-d4 and mycophenolic acid-d3 (MPA-d3).