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Case Report

Pathological and mycological characterization of pulmonary *Aspergillus fumigatus* infection producing gliotoxin in a captive African grey parrot (*Psittacus erithacus*)

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Abstract

Aspergillosis, the main causative agent of which is *Aspergillus fumigatus*, causes mortality in all types of birds. Gliotoxin (GT), one of the multiple virulence factors of *A. fumigatus*, has a variety of immunosuppressive effects. The corpse of an African grey parrot (*Psittacus erithacus*) was sent for necropsy and diagnostic rule-out. The lungs were enlarged, firm, and had dark-red coloration, on the parietal faces of both lungs, some semi-circular caseous necrosis areas were observed. The caudal thoracic and abdominal air sacs were thickened and contained a fibrin-heterophilic exudate. Histopathologically, a necrotic and granulomatous bronchopneumonia was observed with intralesional hyphae with characteristics compatible with *Aspergillus* sp. that were positive with Grocott´s staining. Fibrinous and heterophilic airsacculitis was found in the air sacs. *A. fumigatus* was isolated from lungs, characterized using serial microcultures, and confirmed using polymerase chain reaction. In addition, GT production was detected *in vitro* from the culture filtrate in which the isolate was grown; the organic extract was analysed via thin-layer chromatography. This is the first detection of GT in a case of pulmonary aspergillosis in a parrot, which could help to understand the pathogenesis of the disease in psittacines.

Key words: Aspergillosis, *Aspergillus fumigatus*, gliotoxin, *Psittacus erithacus*.

Introduction

Aspergillosis is a disease of fungal origin that has significant economic implications for the poultry industry; it is also ecologically important, resulting in bird mortality both in the wild and in captivity (22). While this disease is caused by different species of the *Aspergillus* genus, *A. fumigatus* is the predominant cause and is associated with 95% of the cases studied (33). Fungal virulence is multifactorial and includes the production of gliotoxin (GT) (29), a molecule from the epipolythiodioxopiperazine class that has been described in several types of potentially pathogenic fungi (14). GT is characterized by its heterocyclic nucleus, which contains a polysulphide bridge (2-4 sulphur atoms) that has been attributed to several suppressive effects on numerous cell types, particularly macrophages (19), and T and B lymphocytes, where it induces apoptosis and inhibits the activation of nuclear factor kappa-B (NF-kB) (18). The latter effect is an integral part of the immune response and of the regulation of the expression of some inflammatory cytokines (19).

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The study of avian aspergillosis associated with the presence of GT began in the 1990s and was highlighted by the work published by Richard et al. (30), where the toxin was related to the necrotic lung lesions in turkeys infected with *A. fumigatus*. However, studies on this disease and GT biosynthesis in the pathogenesis of aspergillosis in ornamental and pet birds are still scarce. Therefore, the objective of this work is to describe the pulmonary lesions and the detection of gliotoxin in a natural infection caused by *A. fumigatus* in an captive African grey parrot (*Psittacus erithacus*).

Case report

Clinic history.

The corpse of a female African grey parrot (*Psittacus erithacus*), of undetermined age, weighing 440 g, and 1/5 of body condition was sent for necropsy to the Laboratorio de Diagnóstico e Investigación en Enfermedades de las Aves, Departamento de Medicina y Zootecnia de Aves, Facultad de Medicina Veterinaria y Zootenia, Universidad Nacional Autónoma de México (UNAM), was received for necropsy and diagnostic rule-out. Before death, the aviary bird presented with dyspnoea, progressive weight loss, depression and biliverdinuria.

Postmortem findings.

The lesions were located mainly in the lungs; both were enlarged, firm, and had dark-red coloration. These changes involved 90% of the lung parenchyma. On the parietal faces of both lungs, some semi-circular caseous, approximately 1 cm in diameter necrotic areas were observed (Fig. 1). The caudal thoracic and abdominal air sacs were thickened and contained a fibrin-heterophilic exudate. In addition, multifocal necrosis in liver was observed. The aortic trunk was hard and multiple atheromas were seen in the intima that slightly reduced the lumen of the blood vessel. During necropsy, lung, air sacs and liver samples were sampled, fixed in 10% buffered formalin for 24 h, and subsequently processed using routine histological techniques; 3-µm thin-tissue sections were stained with hematoxylin and eosin (H&E) and Grocott stains.

Microscopically, large areas of unencapsulated and poorly demarcated necrosis were observed in the pulmonary parenchyma and exposed bronchi and parabronchi. These necrotic areas were partially surrounded by macrophages and multinucleated giant cells, mixed with heterophilic and fibrinous exudate (Fig. 2). Fungal structures similar to hyphae, which were slightly eosinophilic, septate and possessed 45° dichotomous branches were observed within the necrotic and inflammatory areas of affected lung parenchyma (Fig. 3). The walls of some of the pulmonary blood vessels appeared to be thickened, fragmented, and intensely eosinophilic (fibrinous vasculitis) and contained thrombi. The hyphae were stained black upon treatment with Grocott stain (Fig. 4). In the air sacs, a moderate amount of fibrin intermingled with heterophilic infiltration was observed, but no hyphae were detected in the H&E or Grocott's stained sections. Groups of hepatocytes with multifocal coagulative necrosis interspersed with few heterophils were found, likewise, moderate aggregates of lymphocytes, fibrosis and proliferation of bile ducts were observed around the portal triads.

Fresh lung samples were cultured onto Sabouraud dextrose agar (Bioxon®, Basingstoke, England) supplemented with 0.05 g/L of chloramphenicol (Chloromycetin Pfizer®, Toluca, México) and aerobically incubated at 37 °C for 72 h. A fungal isolate was obtained and its macroscopic and microscopic morphologies were identified using Czapek-

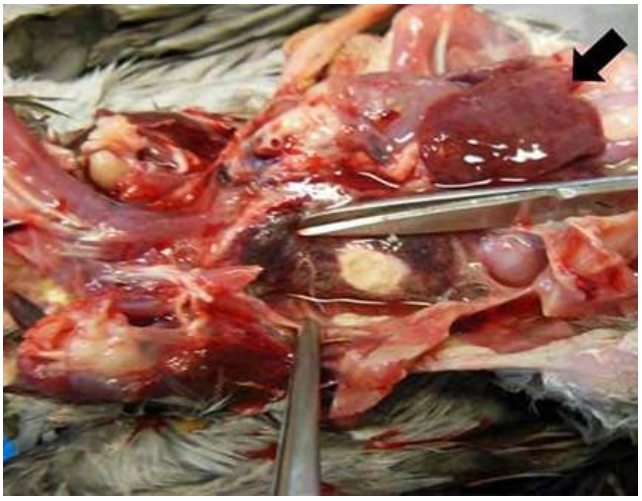

Figure 1. The enlarged right lung has a dark-red coloration, with well- demarcated semicircular area of caseous necrosis extending into the intercostal muscles and ribs. The hepatic atrophy and multifocal necrotic areas are also observed (arrow).

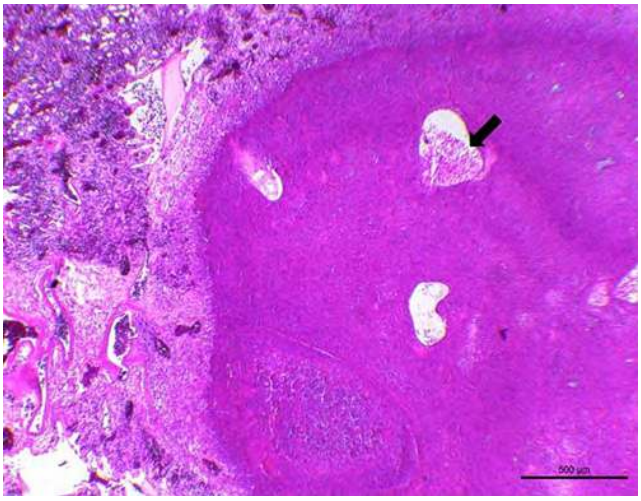

Figure 2. Large necrotic areas intermixed with large aggregates of inflammatory cells and branch fungal structures (arrowhead) in affected lung. HE Bar= 500 µm.

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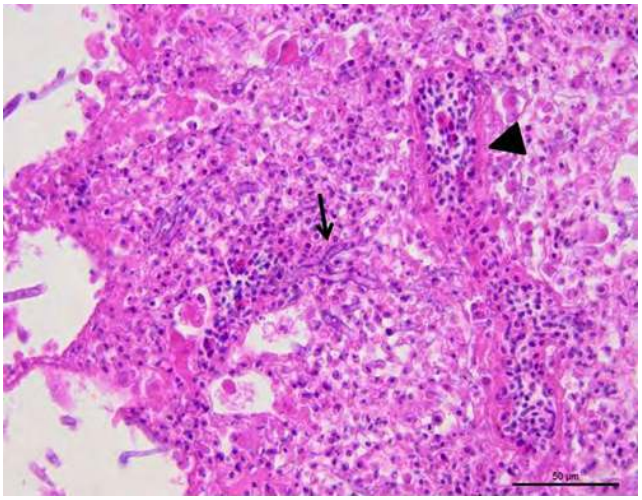


Figure 3. Lung, fibrinoid vasculitis (arrowhead), necrosis and septate hyphae with dichotomous branches (arrow) are observed. HE. Bar = 50 µm.

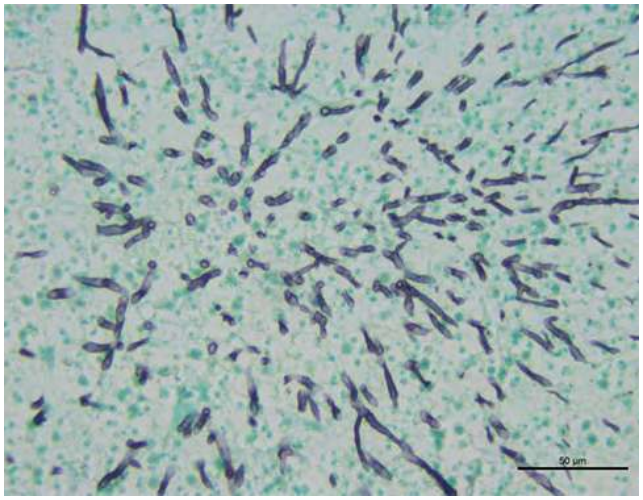


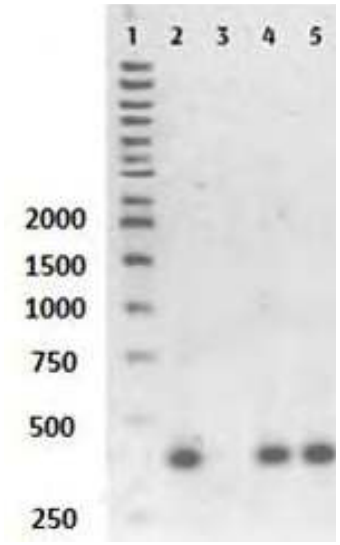
Figure 4. High amount of black, septate hyphae with dichotomous branches in a necrotic area of affected lung. Grocott, 50 µm Bar.

Dox agar (Oxoid®, Basingstoke, England). After serial microcultures at 24, 48 and 72 h performed by the technique previously described by Ridell (32) and stained with lactophenol cotton blue (Hycel®, Naucalpan, México), *A. fumigatus* was isolated and identified.

The DNA extraction from the fungal isolate biomass was performed using a protocol previously published by Mendoza (23). The *A. fumigatus* identification was confirmed by endpoint PCR, amplifying the internal transcribed spacer (ITS)-1-5.1-5.8S-ITS2 fragment, using specific sets of primers for the ITS1 region (Afu1F 5'-GTA TGC AGT CTG AGT TGA TTA T) and the ITS2 region (Afu1R 5'-ATA AAG TTG GGT GTC GGC TG) (8). The amplification products were evaluated by 1% agarose gel electrophoresis (Fig. 5).

In order to perform the GT extraction, the *A. fumigatus* isolate was plated on Czapek-Dox agar (Oxoid®, Basingstoke, England) and incubated at 37 °C for 5 days to recover the conidia with a 1% Tween-80 solution in sterile distilled water. The conidia were counted in a Neubauer chamber, and the inoculum was adjusted to 3 x 106 conidia/ml; 1 ml of the inoculum was added to each one of five Roux bottles with 200 ml of Czapek-Dox broth (Oxoid®, Basingstoke, England). The cultures were aerobically incubated horizontally at 37 °C for 4 days. The biomass was filtered using #1 Whatman® paper (Whatman®, China). The culture filtrate was subjected to two extractions with chloroform at a ratio of 1:8. The chloroform phase was evaporated to dryness at 61.2 °C in a rotary evaporator (11).

The GT analysis was made according to a modified protocol (10). The GT detection was performed via thin-layer chromatography (TLC) using *Gliocladium fimbriatum* GT (Sigma Aldrich®-G9893, St. Louis Missouri, USA) at a concentration of 50 ng/ml as the suggested standard. A standard curve was generated by injecting an aluminium-backed silica gel Chromatoplate (Merck® 60-F254,



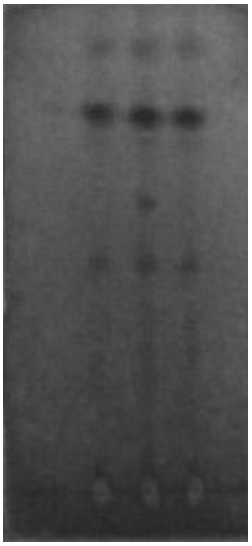


Figure 5 (left). PCR,; 1% agarose gel. Lane 1: 1kb molecular weight marker; lane 2: positive control (*A. fumigatus* A-20); lane 3: negative control (*A. flavus*); lane 4: *A. fumigatus* isolate from the lungs of the African gray parrot; lane 5: Duplicate.

Figure 6 (right). Chromatoplate. Lane 1: *A. fumigatus* organic extract,: gliotoxon (GT) standard (50 ng/ml); lanes 2, 3 and 4 (triplicate): An equivalent GT compound is observed with the same RF as the GT reference.

Germany) with different volumes of the standard (1, 2, 4, 6, 8 and 10 µl). Triplicate samples were analysed on a chromatoplate, where the standard (10 µl) and 10 µl of each sample were injected. The solvent system of the mobile phase was a mixture of dichloromethane and methanol (97:30, 5% v/v of acetic acid). The chromatoplate was observed under UV light at 254 nm to reveal the compound. A compound with the same retention time as the reference GT was detected, and compounds with a different retention time than the standard GT were also observed (Fig. 6).

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Discussion

In this case, *Aspergillus* hyphae associated with necrosis and inflammation were found in the lugs, suggesting that the route of conidia infection was through inhalation, the main infection route in birds (25). In the liver, some hepatocyte groups with multifocal coagulative necrosis were observed intermixed with scarce heterophile. Liver damage has not been associated with aspergillosis, and the observed pathology may instead have had bacterial origin. However, no bacterial isolation was attempted from the liver. The changes described in the aorta suggest that the bird was suffering from atherosclerosis, a disease common to Amazonian parrots and the African grey parrot (5). The reported incidence of this disease in the African grey parrot is 35 to 92%, and given the reported genetic predisposition to atherosclerosis, coupled with breeding and feeding factors, higher incidence of the disease is favoured (4, 15).

Aspergillus infections can induce two types of lesions: (i) granulomatous or nodular, characterized by non-exudative or vascular lesions in the adjacent affected tissue; and ii) diffuse, where edema, fibrin, heterophile, haemorrhage, vasculitis and vascular invasion can be observed in the affected tissue, indicating spread of the infection via another route through the organism (7, 13, 24). However, it is possible to find both types of lesions in the same organ (2, 34), as was observed in our case. In the medicine of ornamental, companion and wild birds, demonstration of combined lesions with the isolation of *Aspergillus* spp. remains the most widely used method for diagnosis. Due to the ubiquity of *Aspergillus* conidia, isolation of the fungi without the presence of lesions is not diagnostic (16). In this case, the histopathologic findings were confirmed by the isolation of *A. fumigatus* from the lungs, and mycological identification was completed by PCR using the ITS1-5.1-5.8S-ITS2 amplifying a fragment from the rDNA complex. Aspergillosis is generally associated with *A. fumigatus*, as in this case; however, *A. niger*, *A. flavus*, A. *nidulans*, *A. glaucus*, and other *Aspergillus* spp. may be associated with this disease (3, 9, 17,26). *A. fumigatus* have small conidia that measure approximately 2 to 3 μm (1) that are not generally trapped in the nasal cavity and the trachea, so they can reach the air sacs and lungs (12). Caudal air sacs (caudal-thoracic and abdominal) are usually considered the primary infection site, but the infection can later spread to the lungs. Once inside the lung parenchyma, the conidia are typically localized to the atrium and parabronchus infundibulum and can be eliminated by phagocytic epithelial cells (24). However, when more conidia accumulate or the bird has a weakened immune system, the innate defence mechanisms cannot eliminate them, and the infection progresses, with subsequent inflammation and necrosis with exudate accumulation in the exposed parabronchus, bronchi, trachea and air sacs (25).

Birds housed in aviaries are more predisposed to develop aspergillosis, due to a high concentration of conidia in the air precipitated by moist, warm, dirty and poorly ventilated environments and long-term storage of food (25, 27, 33). Furthermore, the factors that affect host immunity can also predispose aviary birds to aspergillosis (6).

While the pathogenicity of *A. fumigatus* seems to depend on the immune status of the avian host, the virulence of the isolate is also important. Although GT is not the only virulence factor, it is considered one of the most important (29); therefore, the *A. fumigatus* culture filtrate was evaluated for the presence of GT. Indeed, a compound was detected by TLC with the equivalent retention time (RF) as the reference GT, which represents the first detection of *A. fumigatus* GT in a naturally infected parrot. This helped us to understand the damage mechanisms of this fungus, since the GT was detected in other species has been associated with a wide range of immunomodulatory capacities, inhibiting the phagocytosis of macrophages and the functions of other cells of the immune system and induces apoptosis of host cells (35). Other uncharacterized compounds with lower and higher RF values, unique from the GT standard, were also revealed on the chromatoplate, these other molecules may be aflatoxins or other mycotoxins. According to several studies comparing GT biosynthesis between environmental and clinical isolates, more than 96% of cases, regardless of their origin, are capable of producing the mycotoxin (20). However, it is important to quantify the GT levels to associate its concentration with fungal virulence. This type of quantification has been performed previously in *Galleria mellonella* (greater wax moth) larvae, where some interesting results were revealed. In that study, the authors demonstrated that *A. fumigatus* strains that produce high concentrations of GT are more virulent than isolates with lower biosynthesis capacity (28). According to studies by Richard et al. (30, 31), GT has been detected from the lung tissue of turkeys infected with *A. fumigatus*, both in natural and experimental infections, in which the concentration of the compound in the injured tissue was up to 6 ppm, although this concentration was apparently not related to the infective dose received by the birds, but it was related to the severity of the lesions they presented. On the other hand, GT has also been identified in the lungs and serum of neutropenic mice experimentally infected with *A. fumigatus*, in which the mycotoxin was detected in high concentrations in the tissue (mean, 3976 +/- 1662 ng/g of tissue) and serum (mean, 36.5 +/- 30.28 ng/mL) (21). The methodology described by Lewis et al. (21) to detect GT from the serum of mice was used in cancer patients, in which GT was detected in those patients with invasive aspergillosis (20).

The detection of antibodies against *Aspergillus* by serological methods has not been shown to be sensitive and specific for the diagnosis of aspergillosis (16), but some authors have proposed the use of GT in the diagnosis of invasive aspergillosis by detecting specific antibodies or antigens in the serum (21). Since GT is secreted mainly from the hyphae of *A. fumigatus* (29) during the early stages of the disease, it is possible that it may serve as a diagnostic marker for aspergillosis in pet birds, where it is often underdiagnosed.

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