Systemic Infection by *Aspergillus flavus* in a Mare

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ABSTRACT

**Background:** *Aspergillus* spp. are dimorphic fungus widely distributed in the environment, including in soil, dust and decaying vegetation. Clinical signs of aspergillosis in horses including rhinitis, pneumonia, guttural pouch mycosis, keratomycosis, endometritis, abortions and systemic involvement. In addition, horses with a history of enterocolitis may be predisposed to pulmonary or systemic mycotic infection. However, reports about systemic aspergillosis in horses are restricted to infections by *A. fumigatus* and *A. niger*. There have been no reports of systemic infection caused by *A. flavus* in horses or in other domestic species. Thus, the objective of this work was to describe the epidemiological, clinical and pathological aspects of systemic infection by *Aspergillus flavus* in a mare.

**Case:** A 3-year-old pregnant mare of the Manga Larga, had signs of colic two days prior to admission after grazing in a landfill area to which it had free access. The owner observed remains of plastic bags in the stool. Clinically, there was dehydration, apathy, ocular mucosal congestion, oral cyanosis, reluctance to move, diarrhea, fever, drooling and tachypnea. Due to its clinical condition, the animal was referred to the surgical center for exploratory laparotomy, where compaction was observed in the colon and cecum was verified. Enterotomy and enterolith removal were performed in the small colon region. The mare died after eight days of hospitalization, and necropsy was performed. Macroscopically disseminated lesions were observed in the small colon, stomach, kidneys, lungs, heart and brain. Fragments of tissues from organs in the abdominal and thoracic cavities, as well as from the central nervous system, were collected, fixed in 10% buffered formalin solution, and subsequently routinely processed and stained with hematoxylin and eosin and special histochemical stains to visualize the infectious agent and its morphological characteristics. Samples of lung lesions were submitted to microbiological culture for isolation. Fragments of fungal colonies were conserved in mineral oil for molecular analysis. Histologically, lesions showed acute, necrospurpurative colitis, gastritis, pneumonia, myocarditis, nephritis and meningoencephalitis, associated with vasculitis, thrombosis, infarction and intralesional hyphae that were morphologically compatible with *Aspergillus* spp. The species *A. flavus* was identified based on isolation and polymerase chain reaction analysis.

**Discussion:** A diagnosis of systemic aspergillosis caused by *Aspergillus flavus* was determined by the microscopic examination of lesions associated with characteristic intralesional fungal hyphae and the isolation and identification of the fungal DNA by PCR. A pre-existing disease associated with debilitating factors, including the period of pregnancy, postpartum stress, internment, surgery and the intensive use of antimicrobials and corticosteroids, probably contributed to immunosuppression that favored the proliferation of the infectious agent. Grazing for a long period of time in a place with abundant decomposing organic matter, which is favorable to the proliferation of fungi of the genus *Aspergillus*, may have been a determining factor leading to the infection in the present case as the animal was likely exposed to an excessive number of spores. It is believed that the fungal microorganisms already present in the gastrointestinal tract invaded the surgery-induced lesions present in the mucosa of the small colon and later disseminated to various organs, including heart, lungs, stomach, kidneys and brain. *Aspergillus flavus* can cause enteric infection and hematogenous dissemination in horses, triggering lesions in the heart, lungs, stomach, kidneys and brain, which are characteristic of the systemic form of aspergillosis.

**Keywords:** horse, fungal colitis, systemic aspergillosis, mycosis, immunosuppression.

**Descritores:** equino, colite fúngica, aspergilose sistêmica, micoses, imunossupressão.

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INTRODUCTION

Aspergillus spp. are widely distributed in the environment, including in soil, dust and decaying vegetation [15,21]. Some are important pathogens in humans and animals [20,22]. Among the known pathogenic species, A. fumigatus is the most common, but occasionally, infections by A. flavus, A. niger, A. nidulans, A. deflectus, A. flavipes, and A. terreus are also reported [10]. These fungi are considered opportunistic and are generally associated with depressed immunity associated with intercurrent diseases [2,4] or prolonged administration of antibiotics or corticosteroids [15,21]. Healthy animals are usually resistant but can acquire an infection when exposed to a large amount of conidia [22].

Clinical signs of aspergillosis in horses including rhinitis [17], pneumonia [4,8], guttural pouch mycosis [11,13], keratomycosis [14,16], endometritis, abortions [9] and systemic involvement [10,12,30]. In addition, horses with a history of enterocolitis may be predisposed to pulmonary or systemic mycotic infection [21,24,28]. However, reports about systemic aspergillosis in horses are restricted to infections by A. fumigatus [12,26] and A. niger [5,30]. There have been no reports of systemic infection caused by A. flavus in horses or in other domestic species. Thus, the objective of this work was to describe the epidemiological, clinical and pathological aspects of systemic infection by Aspergillus flavus in a mare.

CASE

A 3-year-old pregnant mare of the Manga Larga breed was forwarded to the Veterinary Hospital of the Federal University of Campina Grande. The owner reported that the animal had signs of colic two days prior to admission after grazing in a landfill area to which it had free access. He observed remains of plastic bags in the stool. Clinically, there was dehydration, apathy, ocular mucosal congestion, oral cyanosis, reluctance to move, diarrhea, fever, drooling and tachypnea. Due to its clinical condition, the animal was referred to the surgical center for exploratory laparotomy, where compaction in the colon and cecum was verified. Enterotomy and enterolith removal were performed in the small colon region. Later, a postoperative treatment was instituted that included meloxicam (Maxicam 2%®) at dose of 0.6 mg/kg, metronidazole (Flagyl®) at 15 mg/kg, flunixin meglumine (Niglumine®) at 0.25 mg/kg, gentamycin (Gentamicina®) at 4.4 mg/kg, ampicillin (Ampicilina Calbos®) at 20 mg/kg, benzathine penicillin (Agrodel Plus®) at 20,000 UI/kg and dexamethasone (Cort-trat®) at 0.15 mg/kg. After 4 days of hospitalization, the mare presented dystocic parturition. The delivery was performed by traction, but the fetus did not survive and was referred for necropsy, which showed no macro or microscopic lesions.

The mare died after 8 days of hospitalization, and necropsy was performed. In the small colon the wall and the adjacent mesentery were thickened, and the mucosa presented a linear area, 5 cm long by 1 cm wide, that was depressed with irregular borders and covered by fibrin. Hemorrhages and multifocal yellowish areas ranging from 0.5 to 0.6 cm in diameter were visualized on the cut surface of the small colon (Figure 1A). In the mucosa of the stomach there were circumscribed multifocal areas (0.5 to 0.6 cm in diameter) that were discreetly elevated and blackened. In the lungs, there were multiple soft yellow nodules (0.5 to 1 cm in diameter) in the parenchyma (Figure 1B). Some of these nodules were protruding on the pleural surface. Multiple yellow and irregular thrombi were observed adhering to the intima of branches of the pulmonary artery (Figure 1B, inset). Multifocal circumscribed areas (0.3 to 0.6 cm in diameter) with a central reddish area and surrounded by a yellow halo were observed in the myocardium (Figure 1C). Multiple thrombi were observed that adhered close to the papillary muscle in the right ventricular endocardium (Figure 1D). In the kidneys, yellowish multifocal areas (0.3 to 0.6 cm in diameter) that were rounded on the cortical surface (Figure 1E) were observed extending from the renal cortex to the medulla. Multifocal areas (0.8 to 1.0 cm in diameter) appearing brown to black, discreetly elevated and penetrating the parenchyma were observed in the frontal and occipital cortex of the brain (Figure 1F).

Samples of lung lesions were seeded on blood agar and incubated at 37°C in aerobic conditions. They were also seeded in 4% Sabouraud dextrose agar with 4% defibrinated sheep blood and MacConkey agar and incubated at 37°C in aerobic conditions. They were also seeded in 4% Sabouraud dextrose agar with...
chloramphenicol and incubated at 25°C for 5 days. Imprints of the microbiological culture were performed and stained with panotic kit (Panótico rápido®). Samples of fungal colonies were conserved in mineral oil for molecular analysis.

The DNA of the isolates was extracted according to the protocol described by Del Poeta et al. [6] and analyzed using polymerase chain reaction (PCR) analysis with oligonucleotides for internal transcribed spacer (ITS) 1 (5’ - TCC GTA GGT GAA CCT GCG G - 3’) and ITS 4 (5’ - TCC TCC GCT TAT TGA TAT GC - 3’), which amplify a product of approximately 600 bp of fungal rDNA ITS1-5.8S-ITS2.

The PCR conditions for a final reaction volume of 25 μL were 20 pmol of each oligonucleotide, 0.2 mM dNTPs, 3 mM MgCl₂, 1x 10x buffer (200mM Tris-HCl, pH: 8.4; 500 mM KCl), 0.2U of Taq DNA polymerase and 10 ng of DNA. The reaction was carried out in thermocycler (MyCycler™ Thermal Cycler) with an initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 57°C for 30 s and extension at 72°C for one min; with a final extension cycle at 72°C for 10 min. Ultrapure water was used as the negative control, and 10 ng of Aspergillus sp. genomic DNA was used as the positive control.

Amplification products of approximately 600 bp were analyzed by electrophoresis in 1% agarose gel, stained with Nucleic Acid Gel Stain (GelRed™), at 10 V per cm, and results were observed with a ChemiDoc™ XRS System using ImageLab software. The obtained amplicons were purified using a GFX PCR DNA and Gel Band kit and sequenced on an ABI-PRISM 3500 Genetic Analyzer. The nucleotide sequences were submitted to GenBank using the BLAST program (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) for identification of microorganisms.

Histologically, lesions were similar in all organs affected, varying in location and intensity. In the mucosa of the small colon there was a focally extensive ulcerated area surrounded by inflammatory infiltrate composed predominantly by neutrophils, many of them degenerate, that extended to the submucosa and was associated with hemorrhage and intrallesional hyphae. Similar lesions were observed in the mucosa of the stomach.

The lungs had multifocal and coalescent areas of necrosis surrounded by marked inflammatory infiltrate composed predominantly by degenerated neutrophils, some lymphocytes and plasma cells and rare macrophages. Intrallesional hyphae, vasculitis and thrombosis in multiple blood vessels, hemorrhages, congestion and moderate intra-alveolar edema were also observed (Figure A and B).

Multifocal areas of infarction containing numerous fungal hyphae were observed in the kidneys and heart (Figure 2C). These areas were surrounded by inflammatory infiltrate composed non-degenerate and degenerate neutrophils and rare macrophages. Fibrin deposition associated with hyphae (thromboembolic glomerulonephritis) was observed in the glomerular tuft. Additionally, the blood vessels presented with vasculitis, fibrinoid necrosis of the vessel wall and multiple thrombi associated with fungal hyphae.

The brain had moderate thickening of the leptomeninges with inflammatory infiltrate consisting of neutrophils distributed mainly around vessels but also extending into the adjacent gray matter. Vasculitis and thrombi were observed in the vessels of the leptomeninges and in the neuropil. Additionally, there were focally extensive areas of infarction with intrallesional hyphae and dilation or vacuolization of the perineurial and perivascular spaces associated with laminar neuronal necrosis and hemorrhages (Figure 2D).

In all affected organs, fungal hyphae were observed mainly in areas of necrosis and/or infarction, thrombi, and blood vessel walls and sometimes free in the parenchyma. In HE-stained samples, the hyphae presented as tubuliform negative images or slightly basophilic and branched. The hyphae were strongly stained black by GMS stain (Figure 2E). In PAS staining, the hyphae presented a strong red color on the walls and sometimes in the cytoplasm (Figure 2F). Morphologically, hyphae were 10-15 μm wide, septate with thin and parallel walls, and showed dichotomous branches at acute angles. Some hyphae also presented ballooning dilatation at the extremities.

In microbiological cultures, the samples were negative for bacteria. In the Sabouraud dextrose agar, after 5 days, were observed the colonies greenish with a granular to cottony aspect (Figure 3A). Microscopically the culture imprints revealed the presence of numerous conidiophores with uni or biserial aspergillar vesicles with abundant phialoconid formation (Figure 3B). The species was identified as A. flavus by microculture method [23]. BLAST sequence analysis of sequences deposited on GenBank showed that iso-
Figure 1. Systemic infection by *Aspergillus flavus* in a mare. A- Small colon cut surface with hemorrhage and multifocal yellowish areas (arrow) and ulceration of covered by fibrin mucosa (arrow head). B- There are multiple yellow nodules distributed across the entire cut surface of the lung. Inset. A verified thrombus (arrow head) in the intima of a large pulmonary vessel. C- Multifocal circumscribed areas with a central reddish area and surrounded by a yellow halo were observed in the myocardium (arrows). D- Thrombi (arrows) are observed adhered to the endocardial surface of the right ventricle. E- Multifocal areas of infarction (arrows) are observed in the kidney surface. F- A focal area of infarct is visualized in the frontal cortex (arrow).
Figure 2. Systemic infection by *Aspergillus flavus* in a mare. A- Lung with areas of necrosis (asterisk) [H&E staining, scale bar= 200 μm]. Inset, numerous fungal hyphae with poorly stained basophilic walls are observed within the necrotic areas. B- Lung with vasculitis and thrombosis (asterisk) with light staining and fungal hyphae (arrows) present in the vascular wall and within the thrombus [H&E staining, scale bar= 50 μm]. C- At the heart there is extensive coagulative necrosis of the myofibrils associated with intralesional fungal hyphae in the cross (arrow head) and longitudinal (arrows) sections [H&E staining, scale bar= 20 μm]. D- Brain with neuropil infarction characterized by perineural and perivascular vacuolization associated with laminar neuronal necrosis (arrow head) and intralesional fungal hyphae surrounded by neutrophils (arrow) [H&E staining, scale bar= 50 μm]. E- Lung with fungal hyphae stained black, with parallel walls, septation and branching at acute angles, sometimes presenting balloon dilatations at their extremities [GMS staining, scale bar= 20 μm]. F- Lung with fungal hyphae strongly stained red [PAS staining, scale bar=20 μm].
late 001 showed 99% (555/559) identity with *A. flavus* (KF434090.1) and isolate 002 showed 97% (555/557) identity with *A. flavus* (JX501356.1).

**DISCUSSION**

A diagnosis of systemic aspergillosis caused by *A. flavus* was determined by the microscopic examination of lesions associated with characteristic intralesional fungal hyphae and the isolation and identification of the fungal DNA by PCR. In this case, conidiophores were not observed in the histopathological analysis, although they are sometimes observed when hyphae are exposed to large amounts of oxygen [18], mainly in cutaneous infections and respiratory tract infections [1,19]. The absence of such structures was probably due to the location of the infectious agent in necrotic lesions with a low oxygen tension.

Grazing for a long period of time in a place with abundant decomposing organic matter, which is favorable to the proliferation of fungi of the genus *Aspergillus* [15], may have been a determining factor leading to the infection in the present case as the animal was likely exposed to an excessive number of conidia. Infections usually occur by inhalation of large numbers of spores or by ingestion [12,27]. In this case, it is believed that the fungal microorganisms already present in the gastrointestinal tract invaded the surgery-induced lesions present in the mucosa of the small colon and later disseminated to various organs. In horses, most of reported infections by *Aspergillus* spp. have been associated with enteric diseases with similar lesions, which consequently favor the invasion of the infectious agent through the mucosa [3,24,28], causing the pulmonary or systemic form of the disease [21,30].

As a disease caused by opportunistic fungi, the determining factors for aspergillosis include conditions that compromise the cellular immunity of horses, such as enterocolitis, sepsis, neoplasms, surgery, disruption of the normal flora due to prolonged antibiotic therapy and the effects of immunosuppressive drugs [15,27]. In the present case, a pre-existing disease associated with debilitating factors, including the period of pregnancy, postpartum stress, internment, surgery and the intensive use of antimicrobials and corticosteroids, probably contributed to immunosuppression that favored the proliferation of the infectious agent.

Thrombi in the right ventricular endocardium and in large pulmonary vessels suggests that the dissemination of the infectious agent occurred via blood flow, causing acute infarctions in the heart, kidneys and brain. These findings are attributed to the angioinvasive characteristics of the genus *Aspergillus* [7,22], which induces vascular injury, thrombi formation and emboli, triggering ischemia and infarctions in various organs [12,18].

Systemic aspergillosis caused by *Aspergillus fumigatus* and *A. niger* is considered uncommon in horses and has rarely been reported [5,12,29,30]. Clinical manifestations of systemic aspergillosis vary according to the affected organs, but it is always a serious disease with an acute clinical course [21]. In this case, the clinical signs observed were associated
with gastroenteric and pulmonary dysfunction without clinical signs involving other systems, probably due to the rapid evolution of the disease.

Histologically, the necrosuppurative inflammatory reaction observed in this case is typical of fungal infections [18], presumably due to acute invasion of the tissues via hematogenous dissemination and the rapid evolution of the disease. In chronic lesions, the inflammatory response may vary from pyogranulomatous to granulomatous [12,26]. The histomorphological and morphotintorial characteristics of the hyphae with parallel walls, diameters of 10-20 μm, septation and dichotomous branching at acute angles [8] are important to the histopathological diagnosis.

Infections by other fungi with histomorphological characteristics similar to Aspergillus, including Pseudallescheria boydii [21], Fusarium spp. [27], zygomycetes [5,29] and also the oomycete Pythium insidiosum [25] should be considered in the differential diagnosis.

CONCLUSIONS

It is concluded that Aspergillus flavus can cause enteric infection and hematogenous dissemination in horses, triggering lesions in the heart, lungs, stomach, kidneys and brain, which are characteristic of the systemic form of aspergillosis.

Declaration of interest. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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