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Retrospective Analysis of Aetiological Agents Associated with Pulmonary Mycosis Secondary to Enteric Salmonellosis in Six Horses by Panfungal Polymerase Chain Reaction

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Summary.

Pulmonary mycosis secondary to enterocolitis is an uncommon diagnosis in equine medicine but is thought to result from mucosal compromise and translocation of enteric fungi. The etiologic agent associated with translocation is often identified based on fungal culture or hyphal features in histologic sections. In order to better understand the etiologic agents involved, 6 horses diagnosed with *Salmonella* enteritis and concurrent pulmonary mycosis were retrospectively identified through a database search of a veterinary teaching hospital records. The cases were subjected to PCR and sequencing of the internal transcribed spacer 2 (ITS-2) located between 5.8S and 28S rRNA genes to identify the etiologic agent involved. Sequencing identified *Aspergillus fumigatus*, *Aspergillus flavus*, *Fusarium* sp., *Cladosporium* spp., and *Curvularia* sp. A single case had a dual infection with *Fusarium* sp. and *Aspergillus fumigatus*.

Introduction

Pulmonary mycosis is uncommonly diagnosed in equine medicine and is associated with either inhalation of conidia or translocation of gastrointestinal microorganisms following compromise of the mucosal barrier (Blomme, 1998). Pulmonary translocation of enteric fungi subsequent to enterocolitis has been documented after infection with *Salmonella* spp. or *Neorickettsia risticii*, or idiopathic causes of enteritis (Breshears *et al.*, 2007; Hattel *et al.*, 1991; Rosenstein and Mullaney, 1996; Slocombe and Slauson, 1988; Sweeney and Habecker, 1999). The diagnosis of pulmonary mycosis is often made at the time of necropsy as horses rarely demonstrate clinical signs. Even in horses with clinical signs of respiratory disease such as tachypnea, increased respiratory effort, or crackles, diagnosis through trans-tracheal wash or thoracentesis is difficult (Sweeney and Habecker, 1999).

The pathogenesis is likely multifactorial; contributing factors include neutropenia, immunosuppression, or endocrinopathy (Carrasco *et al.*, 1996; Hattel *et al.*, 1991; Pace *et al.*, 1994). Enteric pathogens like *Salmonella* spp. cause disruption of the mucosal barrier, which presumably allows microorganisms from the normal gastrointestinal microbiota to invade from the GI tract to the lung through the circulatory system. The exact mechanism behind

Conflict of Interest

The authors declare no conflicts of interest with respect to the publication of this manuscript.

this phenomenon has not been elucidated and remains somewhat speculative. Many horses with enterocolitis do not develop pulmonary mycosis despite severe compromise of the mucosal barrier and thus an exact correlation of breach of the mucosal barrier with translocation of fungi cannot be stated.

The pulmonary lesion associated with enteric translocation centers on the pulmonary vessels and is composed of necrosis and fibrin deposition with large mats of fungal hyphae surrounded by an intense inflammatory infiltrate with neutrophils and macrophages (Breshears *et al.*, 2007; Slocombe and Slauson, 1988; Sweeney and Habecker, 1999). Most cases are presumptively diagnosed as aspergillosis based on hyphal morphologic features in histologic sections or fungal culture (Breshears *et al.*, 2007; Hattel *et al.*, 1991; Slocombe and Slauson, 1988; Sweeney and Habecker, 1999). Histologic features often do not provide a definitive etiologic agent; therefore, diagnosis requires ancillary methods like fungal culture, polymerase chain reaction (PCR), immunohistochemistry, or immunofluorescence (Kaufman, 1992).

In order to better elucidate the etiologic agents associated with translocation of enteric fungi, a retrospective study of horses diagnosed with both *Salmonella* enteritis and pulmonary mycosis was performed via molecular analysis of the panfungal ITS-2 region.

Materials and Methods

Experimental Design

Archived records from a veterinary teaching hospital were queried for a combination of the terms “salmonellosis” or “*Salmonella*” and “pulmonary mycosis” or “fungal pneumonia” to identify cases of *Salmonella*-induced enterocolitis with secondary pulmonary mycosis. Only cases which had confirmed salmonellosis via culture or PCR and histologically diagnosed fungal elements in lung lesions were included.

Salmonella Culture and Molecular Testing

Culture for *Salmonella enterica* for cases 1–5 was performed by inoculating the patient specimen in tetrathionate enrichment broth (Difco, Becton Dickinson, Franklin Lakes, NJ) and incubating it overnight at 37°C followed by inoculation onto xylose-lysine-tergitol (XLT-4; Becton Dickinson) agar and MacConkey agar (Becton Dickinson). Colonies on XLT4 agar consistent with *Salmonella* morphology were subcultured to trypicase soy agar supplemented with 5% sheep blood (blood agar plate; Becton Dickinson), incubated overnight at 37°C. *Salmonella* spp. were identified based on characteristic biochemical reactions when grown on triple sugar iron agar (TSI; BD), lysine iron agar (LIA; BD), Christensen’s urea agar (BD), sulfide, indole, motility medium (SIM; BD), and agglutination with polyvalent antisera (BD). For case 6, polymerase chain reaction (PCR) was used to determine the presence of the *Salmonella spaQ* gene in the feces as previously described (Kurowski *et al.*, 2002).

Fungal Culture

For case 6, tissue collected at necropsy was submitted for fungal culture. Tissue samples were used to inoculate Sabouraud dextrose agar (BD) and potato dextrose agar (BD). Cultures were incubated at room temperature for up to three weeks and examined every three days for the presence of fungal organisms.

PCR Analysis

Fifty micrometers curls of formalin-fixed, paraffin-embedded (FFPE) lung tissue from each case was used for PCR analysis. DNA extraction, fungal amplification targeting the ITS-2 region, and sequencing was performed as previously described (Meason-Smith *et al.*, 2017). Amplification and sequencing of the ITS-2 region (ITS3/4) was performed using primers ITS3 (5'-GCATCGATGAAGAACGCAGC-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3' (White *et al.*, 1990).

Results

All of the cases were diagnosed with diarrhea (6/6) with varying lengths of duration from 7 days to one month (Table 1). Diagnosis of *Salmonella* was made by culture (5/6) and PCR (1/6) of fecal samples collected either during hospitalization or at the post-mortem examination. Clinical signs of respiratory disease were only reported in 2 of the cases (Table 1). Five of the six cases had complete blood counts available for review, and mild neutropenia was noted in the early time course in three cases (Table 1).

A complete necropsy was performed in all cases, and tissue samples were fixed in 10% neutral buffered formalin prior to processing. Postmortem findings included fibrinonecrotizing and ulcerative typhlocolitis (2/6), fibrinoulcerative colitis (3/6), fibrinous peritonitis (2/6), ulcerative enteritis (1/6), embolic granulomatous nephritis (1/6), embolic pneumonia (6/6), fibrinous pleuritis (1/6), and jugular vein thrombosis (1/6). The lungs were edematous with multifocal embolic tan, variably sized, raised nodules surrounded by a red reactive rim (Fig 1).

Histologically, the lungs of all cases (6/6) had areas of infarcts, characterized by necrotizing and neutrophilic vasculitis with fibrin thrombi and hemorrhage (Fig 2). Fungi were sometimes observed in the vessel wall or within the vessel lumen (Fig 2). Adjacent pulmonary parenchyma had varying degrees of necrosis surrounded by intact and degenerate neutrophils and, in select cases (1,3,5), epithelioid and multinucleated macrophages. The inflammatory infiltrate in case 1 was composed of a mixed inflammatory population of Langhan's and foreign-body type multinucleated giant cells (Fig 3) and degenerate neutrophils; macrophages occasionally contained phagocytosed fungal elements. The hyphae in case 1 had pigmented, septate, 6–8 μm walls admixed with 7–20 μm yeast-like bulbous swellings that occasionally formed monoliforme hyphae. In 3 cases (2,4,6), hyphae were 3–6 μm with parallel walls, infrequent septation, and acute angle dichotomous branching centered on the blood vessels (Fig 4); in case 6, 4 μm globose vesicles with radiating pale yellow uniseriate sterigmata covered by numerous 1 μm, round basophilic

conidia were seen within alveolar spaces (Fig 4 inset). Case 3 and 5 hyphae had 4 μm parallel walls with infrequent septation and acute angle branching.

Fungal culture for case 6 isolated *Fusarium* sp. All of the cases were ITS-2 PCR positive. Sequence analysis identified 1 case of *Curvularia* sp. (case 1), 2 cases with *Cladosporium* sp. (case 3,5), 1 case of *Aspergillus flavus* (case 2), 2 cases *Aspergillus fumigatus* (cases 4,6), and 1 case of *Fusarium* sp. (case 4) (Table 2).

Case 6 invited increased scrutiny because an initial PCR amplification and sequencing identified *Aspergillus fumigatus*, whereas fungal culture from the same tissues isolated *Fusarium* sp. In order to better understand the discrepancy between culture and PCR results, a second PCR amplification and sequencing was attempted, which matched *Fusarium* sp. Furthermore, a real-time PCR reaction using the same primers identified two peaks on the melting curve suggesting a dual infection. This was further supported by histological analysis, which identified characteristic *Aspergillus* spp. conidiophores and fungal hyphae consistent with both species based on patterns of 90 degree (*Aspergillus* spp.) and acute angle branching (*Fusarium* sp.).

Discussion

PCR amplification of the ITS-2 region identified a specific etiologic agent in all 6 cases of pulmonary mycosis. Similar to previous reports of pulmonary mycosis secondary to enterocolitis, *Aspergillus* spp. were the most commonly identified etiologic agents (Slocombe and Slauson, 1988; Sweeney and Habecker, 1999). However, *Curvularia* sp., *Cladosporium* sp., and *Fusarium* sp. were also identified. These fungal agents are all considered opportunistic angioinvasive pathogens (Perusquia-Ortiz *et al.*, 2012).

Fibrinonecrotizing enterocolitis is a well-described feature of salmonellosis (Uzal *et al.*, 2007). The cases reported herein had a range of morphological diagnoses, but a common feature was mucosal necrosis that lead to ulceration and loss of the mucus layer and epithelial barrier. In health, the mucus layer, tight junctions of the epithelium, resident submucosal macrophage population, and gut microbiota work synergistically to prevent pathogens from creating disease (Brenchley and Douek, 2012). However, *Salmonella* and other enteric pathogens secrete enterotoxins, which cause severe disruption of the mucosal barrier that could allow fungi and bacteria that exist in the equine gut to translocate beyond the lumen (Brenchley and Douek, 2012).

Salmonellosis often causes neutropenia, which diminishes the host's immune response (Manship *et al.*, 2019; Uzal *et al.*, 2007). In the three horses with mild neutropenia (cases 1–3), no correlation was found with etiologic agent recovered by PCR. Interestingly, in a mouse model of microbial translocation secondary to mucosal compromise, decreasing neutrophil number alone was insufficient to result in microbial translocation (Koh *et al.*, 2008). However, when the mucosal barrier was disrupted and the neutrophil population was diminished, 100% of the mice developed microbial translocation and systemic disease (Koh *et al.*, 2008). This suggests that the pathogenesis of enteric translocation and pulmonary mycosis is multifactorial and requires a combination of neutropenia, immunosuppression,

and mucosal barrier disruption. Previous reports of pulmonary mycosis secondary to salmonellosis have reported neutropenia of varying degrees (Slocombe and Slauson, 1988; Sweeney and Habecker, 1999). Reports in the medical literature of invasive fusariosis and aspergillosis associated with lung involvement often involve immunocompromised or immunosuppressed individuals (Fraser, 1993; Nucci and Anaissie, 2007).

The ability of the immune system to respond to an infection is often reflected in the type of histologic lesion that arises in the lung (Berenguer *et al.*, 1995; Stergiopoulou *et al.*, 2007). In an experimental model of invasive pulmonary aspergillosis, neutropenic mice have a more angiocentric lesion with hemorrhage and pulmonary infarction similar to what is seen in horses with neutropenia secondary to salmonellosis (Berenguer *et al.*, 1995; Sweeney and Habecker, 1999). In contrast, non-neutropenic mice were reported to have a greater inflammatory response without angioinvasion (Berenguer *et al.*, 1995). Similarly, in a study of human patients with angioinvasive pulmonary disease, neutropenic individuals had a more significant histologic lesion than the non-neutropenic cohort (Stergiopoulou *et al.*, 2007). These studies both evaluated inhalational angioinvasive aspergillosis, so caution must be used in applying conclusions from this study to the present cases. However, the findings are still intriguing in the context of pulmonary mycosis following enterocolitis because the lesion in the equine lung tends to be centered on blood vessels, and salmonellosis leads to neutropenia to varying degrees.

A definitive causation between salmonellosis and microbial translocation has not been established, so it is worth noting that the agents identified may have arrived in the lung via translocation of enteric microbiota or inhalation of environmental spores. *Aspergillus* spp., the most commonly identified etiologic agent in this study, is thought to cause respiratory disease in horses primarily from inhalation of conidia (Fraser, 1993). Similarly the rare reports of pulmonary disease caused by *Curvularia* and *Cladosporium* spp. involve spores that act as allergens and contribute to chronic obstructive pulmonary disease (Costa *et al.*, 2006). Since respiratory signs are not uniformly seen in horses with pulmonary mycosis secondary to translocation, it is possible that the actual incidence of this condition is underestimated, which contributes to the ambiguity of the pathogenesis (Slocombe and Slauson, 1988; Sweeney and Habecker, 1999).

The etiologic agents identified in this study are intriguing, as three species were identified that have not been previously reported as causes of pulmonary mycosis secondary to salmonellosis. This suggests that the intrinsic angioinvasive properties of *Aspergillus* spp. alone cannot explain the phenomena of translocation. In humans with severe immunosuppression from HIV and in mouse models of mucosal compromise, *Candida albicans* is the most common etiologic agent responsible for microbial translocation (Koh *et al.*, 2008; Marchetti *et al.*, 2013). Since this study did not identify *Candida albicans*, it is possible that this species is not abundant in the equine gastrointestinal tract. Unfortunately, the equine gastrointestinal microbiome remains to be sequenced, and it is unknown if the fungi identified in this study are components of the normal gastrointestinal mycobiota.

Conclusions

Aspergillus spp. were previously thought to be the only cause of pulmonary mycosis secondary to salmonellosis; however, the use of panfungal PCR in our study identified novel etiologic agents for this condition. The dual infection of case 6 highlights the potential for multiple pathogens to be involved in generating pulmonary disease secondary to translocation.

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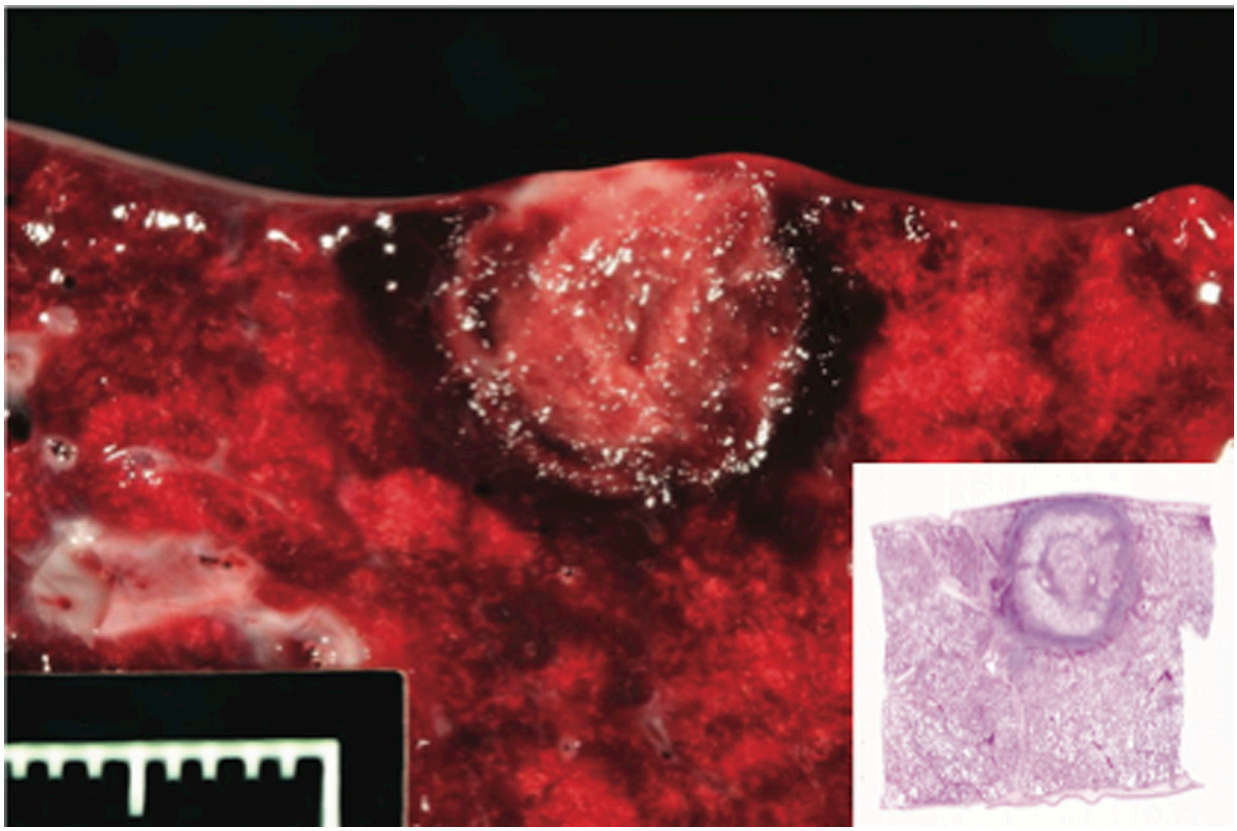

Figure 1. Pulmonary mycosis secondary to enteric translocation of fungi.

Case No. 6. Cross section of the pulmonary parenchyma highlights the vasocentric nature of the lesion. Scale bar= 1 cm **Inset.** Low magnification of lung. Hematoxylin and eosin (HE).

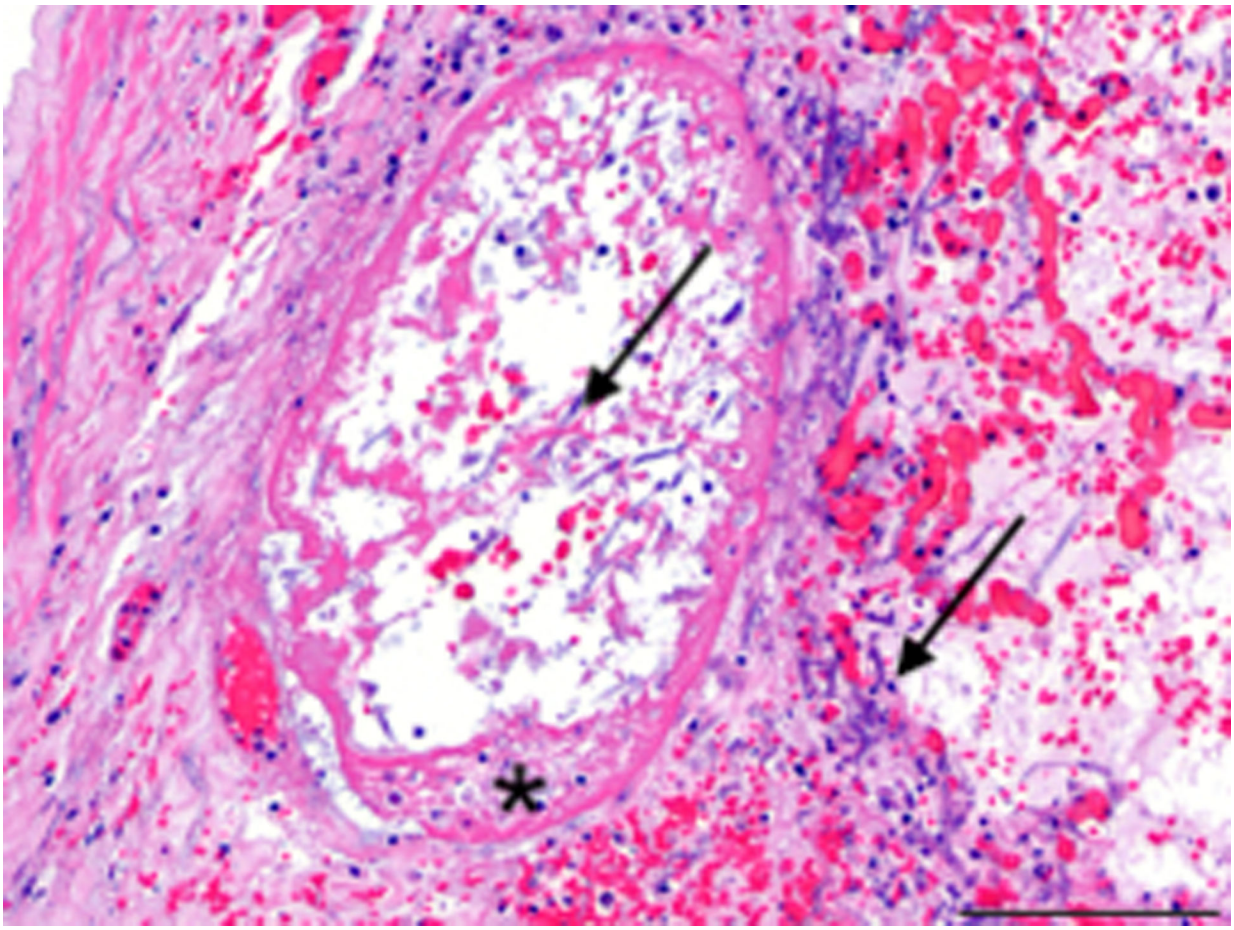

Figure 2. Pulmonary mycosis secondary to enteric translocation of fungi.

Case No. 2. (*Aspergillus flavus*) Fibrinonecrotizing vasculitis (*) surrounded by hemorrhage and intense inflammatory infiltrate of neutrophils and macrophages. Fungal hyphae are within the vessel lumen and in areas of inflammation and hemorrhage (arrows). HE, Bar=100 μm

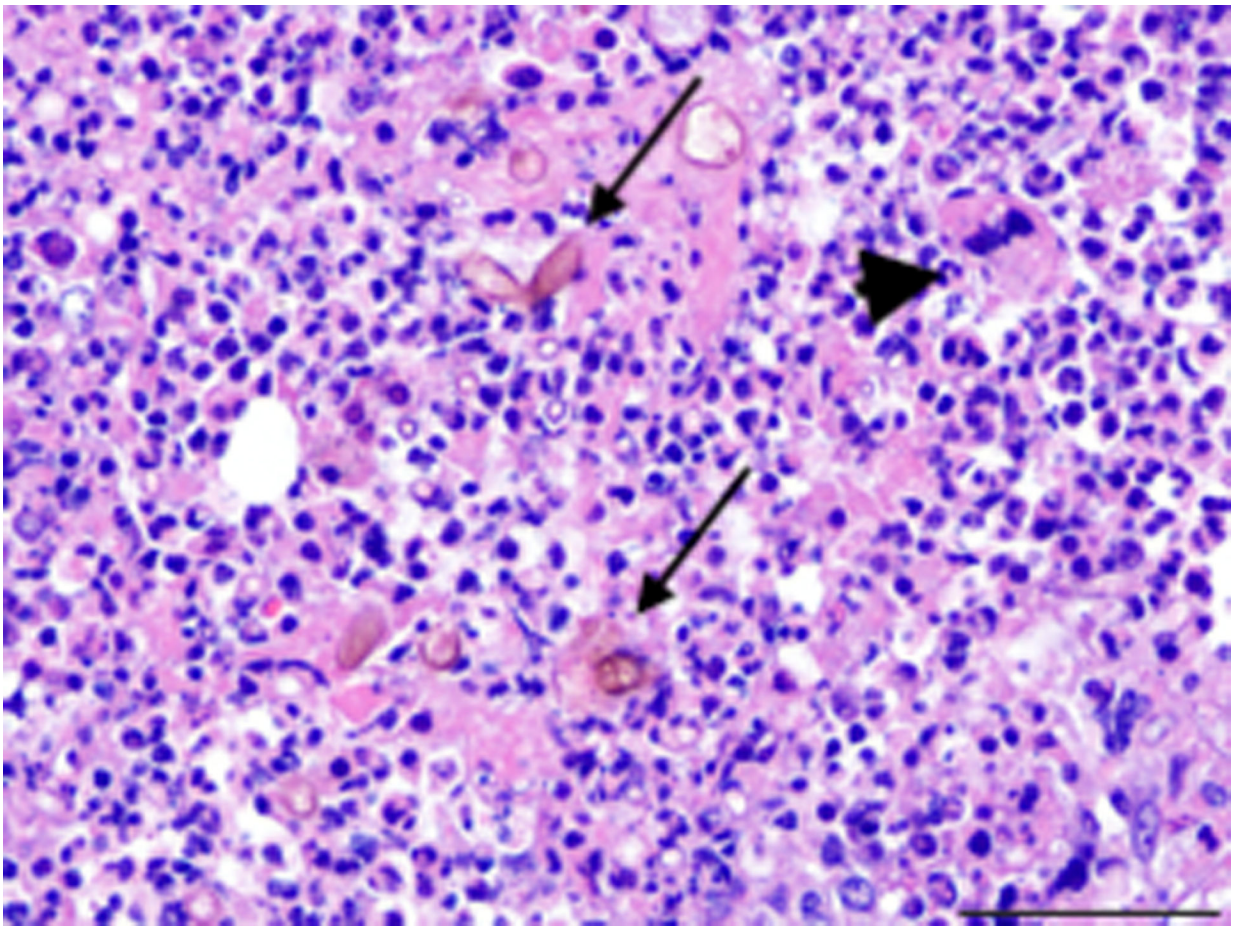

Figure 3. Pulmonary mycosis secondary to enteric translocation of fungi.

Case No. 1. (*Curvularia spicifera*) A mixed inflammatory population of multinucleated giant cells (arrow heads) and degenerate neutrophils. Multinucleated macrophages occasionally contain and surround phagocytosed 6–8 μm walls hyphae with 7–20 μm yeast-like bulbous swellings (arrows) HE, Bar=50 μm.

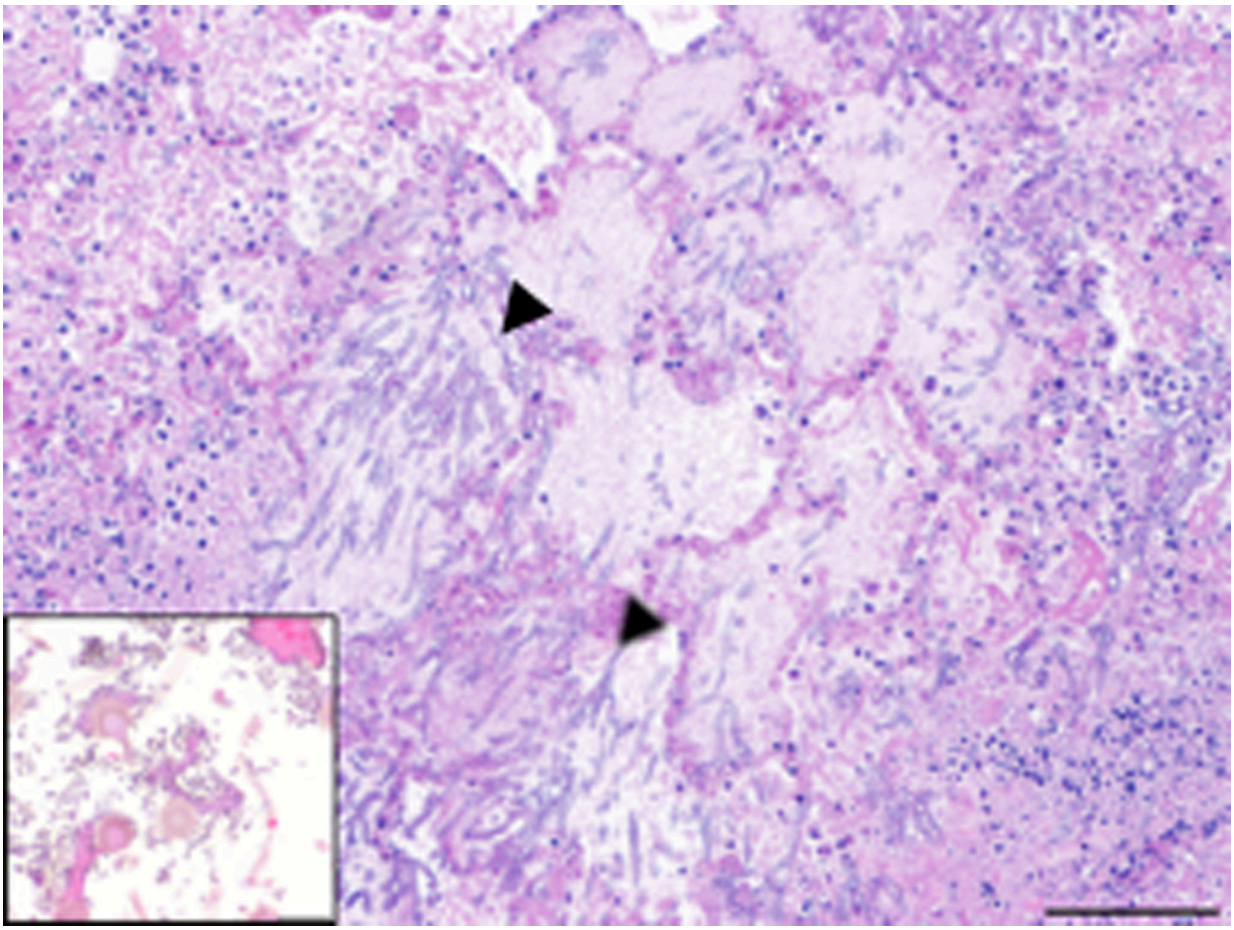

Figure 4. Pulmonary mycosis secondary to enteric translocation of fungi.

Case No. 6. (*Aspergillus fumigatus* and *Fusarium oxysporum*) Neutrophilic and histiocytic pneumonia with mats of fungal hyphae that have 3–6 μm parallel walls, infrequent septation, and acute angle dichotomous branching (arrow heads). HE, Bar= 200 μm. Inset. 60x magnification of *Aspergillus fumigatus* conidia. HE, Bar= 20 μm.

Table 1.

Case signalment, history and ancillary diagnostics.

Case Number	Signalment	History	Morphologic Diagnoses	Ancillary Diagnostics
1	6-year old Quarter Horse mare	Colitis with rectal prolapse Laminitis Labored respiration	Fibrinous peritonitis Ulcerative enteritis Fibrinonecrotizing typhlocolitis Granulomatous nephritis Embolic pneumonia with hemorrhage	Postmortem culture of colon: *Salmonella* positive
2	6-month old Quarter Horse filly	Cough and nasal discharge Diarrhea, anorexia, fever	Fibrinonecrotizing typhlocolitis Embolic pneumonia with fibrinous pleuritis	Antemortem fecal culture: *Salmonella* positive
3	1.5-year old Quarter Horse stallion	Puncture wound Diarrhea Acute renal failure Laminitis	Fibrinoulcerative colitis Laminitis with rotation of P3	Postmortem culture of colon: *Salmonella* positive
4	12-year old Quarter Horse gelding	Diarrhea for 1 week	Fibrinonecrotizing and hemorrhagic colitis Embolic pneumonia	Postmortem culture of colon: *Salmonella* positive
5	4-month old Appaloosa filly	Diarrhea for 1 month	Chronic ulcerative colitis Left jugular vein thrombosis Embolic pneumonia	Postmortem culture of colon: *Salmonella* positive
6	5-year old Quarter Horse gelding	Diarrhea, fever Ataxia, hindlimb weakness, horizontal nystagmus (hyperammonemia)	Fibrinous peritonitis Fibrinoulcerative and necrotizing colitis Embolic pneumonia with hemorrhage	Fecal PCR: *Salmonella* positive; Clostridial toxins: negative; Fungal culture of lung: *Fusarium* sp.

Table 2.

Morphologic diagnoses and results of fungal ITS-2 DNA sequencing in 6 horses with pulmonary mycosis.

Case number	Aetiological agent	Fungal morphology	Homology	NCBI GenBank accession number
1	*Curvularia* spp.	Pigmented, septate, 6–8 μm walls; 7–20 μm yeast-like bulbous swellings	100%	KU729101
2	*Aspergillus flavus*	3–6 μm with parallel walls, infrequent septation and acute angle dichotomous branching	100%	MH862262.1
3	*Cladosporium* spp.	4 μm parallel walls with infrequent septation and acute angle branching	98%	MH875399.1, MH865597.1
4	*Aspergillus fumigatus*	3–6 μm with parallel walls, infrequent septation and acute angle dichotomous branching	98%	KU319436
5	*Cladosporium* spp.	4 μm parallel walls with infrequent septation and acute angle branching	99%	MH865597.1, MH863870.1
6	*Aspergillus fumigatus*; *Fusarium oxysporum*	3–6 μm with parallel walls, infrequent septation and acute and 90-degree angle dichotomous branching	100%; 100%	MH185963, KY318502