Keeping them out of the rough: Practical Insights into Hemorrhagic Bowel Syndrome

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Abstract

Hemorrhagic bowel syndrome (HBS) is newly-described disorder primarily affecting dairy cattle. Its cause is not known. In this study, the possibilities that *Aspergillus fumigatus* and *Clostridium* were involved in etiology of HBS were examined. Samples of feed, gastrointestinal (GI) contents, GI wall, lymph nodes and blood from HBS and control animals were collected in four states (IA, ID, OR, WA). Real-time SybrGreen quantitative polymerase-chain reaction (PCR) analysis indicated that all HBS cows were infected with *A. fumigatus*. Samples from control cows were negative. Multiplex PCR analysis of five clostridial toxin genes did not reveal a correlation with HBS. Specifically, clostridial toxin genes were detected in both HBS and control animals. *A. fumigatus* correlates closely with HBS and may play an important role in its etiology.

Introduction. In 1991, Anderson reported a new disease in Idaho dairy herds (1). The syndrome, which he termed "Point Source Hemorrhage", was observed in five high-producing Holstein cows from one dairy. Symptoms included point-source sub-mucosal hematomas, each affecting 10-20 cm of the jejunum. One of the five cows exhibited a ruptured hematoma with exsanguination into the lumen of the jejunum. The origin of the hematoma was traced to the jejunal submucosa which dissected mucosa from underlying connective tissue. Despite the hemorrhage, clotting time was normal. No bacteriological assays proved definitive and no ulcerative processes, parasitism or vaculitis were apparent. Feeding and management practices on the dairy were "exemplary".

Incidence. Since 1991, awareness of "Point Source Hemorrhage" (now more commonly known as "**Hemorrhagic Bowel Syndrome (HBS)**"), in dairy cattle has grown. Dennison *et al* (2) published a survey of HBS in 22 dairy cows in 2002. Dr. Bruce Anderson, in his practice with the University of Idaho in Caldwell, typically conducts necropsies on 10-20 cases per year and, in one dairy, has noted 35 cases since 1985. HBS incidence is increasing (3,4) and is responsible for 2% of the deaths of dairy animals in the US (5). Additional estimates of incidence may be unavailable because there is a marked seasonality to the disease (more cases occur in cooler winter months), many veterinarians, dairy producers and nutritionists are unfamiliar with the disease, symptoms mimic common ruminant digestive diseases and an unknown proportion of afflicted cattle are submitted for necropsy.

Signalment and etiology. HBS is characterized by sudden drop in milk production, abdominal pain due to obstructed bowel and anemia (6). Death comes within 48 hours from the onset of the obstructing blood clot plug. Fatal factors are presumed to be the anemia combined with digesta stagnation in much of the severely dilated small intestine proximal to the plug (6).

What is the cause of HBS? Dennison *et al* (2) conducted a retrospective analysis of all dairy cows examined at Colorado State University Veterinary Teaching Hospital which were submitted with dysentery, melena or colic. Cows with hemorrhagic enteritis were considered to have HBS if they displayed melena and clotted blood in feces or small intestine and displayed no evidence of intestinal or extra-intestinal lesion. Of the 22 HBS cows, age ranged from 2-8 years and incidence occurred at a mean of 107.5 days post-parturition. Average milk production was 89.8 lbs. Two-thirds (14 of 22) of the cases arrived in the cooler months (September through February). Lethargy was noted in 21 of 22 cows. Abdominal distension was noted in 8 of 22. Nineteen of 22 were clinically dehydrated and 10

of 17 displayed ruminal hypomotility. Seven of 15 had bloody feces. Distended loops of the small intestine were noted in 7 of 14 cows and trans-abdominal ultrasonography revealed small intestinal ileus and distension in 12 of 12 cows. Seven of 8 cows treated medically died and nine of 13 cows treated with surgery died or were euthanized. *Clostridium perfringens* was isolated from fecal samples in 17 of 20 cows. Genotyping of the *C. perfringens* in 10 cows revealed Type A in five cows and Type A with the b2 toxin gene in the remaining five cows. The dairy from which the *C. perfringens*-positive cows had originated had vaccinated the cows with *C. perfringens* types C and D toxoid.

Despite finding *C. perfringens* in most HBS cows, Dennison *et al* (2) commented that "it is unclear whether proliferation of *C. perfringens* is part of the primary disease process in cows with HBS or occurs as a secondary response." Evidence against *C. perfringens* playing the primary etiologic role includes the observations that *C. perfringens* is ubiquitous (7,8). Furthermore, immunization against *Clostridium* spp. does not appear to protect animals from HBS.

Many potential causes of HBS have been investigated and discarded. Agents which do not play an etiologic role include parasitism (1), BVD, coccidia, salmonella, coagulopathies, intestinal foreign bodies, physical obstructions and deformities including volvulus and intussuception (2). Furthermore, analyses of diets, ages of cow, levels of milk production and a full spectrum of blood chemistry and biochemical assays failed to reveal a consistent clinical correlate to HBS (2).

An alternative etiology. Moldy feed was observed on several dairies which experienced cow losses due to HBS. In "human literature" *Aspergillus fumigatus* is described as pathogenic, causing intestinal bleeding and invasive apsergillosis in immunocompromised patients. We hypothesized that immunocompromised dairy cows might develop HBS when exposed to *A. fumigatus* –laden feed.

Pathogenicity of *Aspergillus fumigatus.* *Aspergillus* is a large fungal genera containing > 100 species. Of these, *A. fumigatus* and *flavus* are the most pathogenic (9,10). Pathogenicity of *Aspergillus* is attributed to three virulence factors: 1) production of iron (Fe)-sequestering siderophores, 2) secretion of complement- and phagocytic-inhibitory lipids and 3) secretion of proteases (9,10). Specifically, *A. fumigatus* is able to meet its Fe requirement, and thereby maintain growth, by the actions of its proteolytic enzymes. These liberate the host's Fe stores from transferrin and lactoferrin and allow Fe transfer to triacetylfusarimine and ferriciocin (10). In addition, invasiveness is facilitated by secretion of polar and neutral lipids, phenolic compounds and heterocyclic toxins (including aflatoxins and other toxins). Some of these inhibit phagocytosis while others suppress the immune response of the host by inhibiting complement factors C3a and C5a (10). Finally, pathogenic species of *Aspergillus,* like an invasive tumor, secrete proteases which facilitate hyphal penetration from a colonization site into the underlying parenchymal tissue (10).

***Aspergillus* in the ruminant gut.** Several studies have demonstrated potential for *Aspergillus* species to infect the ruminant gut at various sites and to cause enteric hemorrhage. Sheridan (11) reported aspergillosis in calf abomasum in 1981. In 1989, Jensen *et al* (8,12) reported that *A. fumigatus* infected the terminal gastric compartments, particularly the omasum. In 1991, Jensen *et al* (13) received a 4 year-old Jersey cow with suspected right-displaced abomasum (RDA) and acidosis. The RDA and acidosis were <u>not</u> confirmed by examination. The animal also did <u>not</u> respond to antibiotics or anti-inflammatory drugs. The animal was euthanized and necropsy revealed hemorrhagic lesions in the reticulum, rumen, omasum, and small intestinal Peyer's patches. Hemorrhagic necroses of the mesenteric lymph nodes, liver, kidney and lung were observed. Hyphal growth, thrombosed vessels and vasculitis were detected in all necrotic tissues. *A. fumigatus* and *A. corymbifera* were identified in all necrotic lesions. Jensen *et al* (13) surmised that the ruminant gut provided two portals for fungal

invasion: intestinal Peyer's patches and the pre-gastric digestive compartments and proposed that *A. fumigatus* was the primary invader.

In more recent studies (14,15) Jensen *et al* evaluated predisposing factors for mycotic infections in ruminants. The most common mycoses included aspergillosis, candidosis and zygomycoses. Principle etiologic agents were *A. fumigatus*, *Candida albicans* and *Absidia corymbifera*, respectively. *Mucor pusillus* and *Rhizopus* spp. were also identified a common etiologic agents in zygomycoses (14). Portals for infection were identified in the respiratory and GI tracts. GI mycosis was identified with the omasum representing the main organ for infection. In one study (15), 32 of 694 cattle submitted for necropsy had gastrointestinal mycoses with an elevated incidence in cooler months (i.e., 75% incidence was in October through March, a time during which stored feed is fed to cattle). Hypomotility of the foregut was noted in 22 of 32 cattle, 23 of 29 cattle were post-partum, 22 of 29 had been given broad-spectrum antibiotics, 9 or 29 had been given anti-inflammatory drugs, 26 of 29 displayed inappetance, 23 of 29 displayed diarrhea and 17 of 29 displayed fever. *Aspergillus* and zygomycetes were detected in gut wall vasculature with thromboses and vasculitis. Hematogenous spread of fungi to the liver, lung and kidney was detected. *A. fumigatus* was detected in 10 of 21 cows, *A. corymbifera* was detected in 8 of 23 cows and Candida was detected in 1 cow. Of interest, animals were never infected with more than one fungal species. Predisposing factors for mycotic infections included: **1)** feeding of moldy feed, **2)** immunocompromizing diseases, **3)** acidosis, **4)** anti-microbial therapy, **5)** reflux of abomasal contents, **6)** metabolic disturbances, **7)** post-partum stress, **8)** viral erosive diseases such as IBR, **9)** anti-inflammatory treatment, and **10)** abortion.

Asperillosis in immunocompromised humans. *A. fumigatus* is ubiquitous. Yet it rarely causes serious disease in healthy individuals. Immunoincompetence is the primary predisposing factor in *Aspergillus* infection (invasive aspergillosis; 16-19) in humans. Patients with AIDS, cancer and those receiving organ transplant are particularly susceptible to invasive aspergillosis (17-19). For example, invasive aspergillosis occurs in 2.6 – 10.3% of all bone marrow transplant patients and has a mortality rate of 56 to 88.1% (16).

Immunosuppression in dairy cows. Mallard *et al* (20) have reported that immunosuppression is common in dairy cows and accounted for the high incidence of disease. Changes in both immune function and nonspecific host defense mechanisms have been reported in dairy cows at onset of lactation (21-25). Stressors in lactation include **1)** a high energy diet (and potential acid reflux), **2)** ketosis, **3)** milk fever, **4)** lameness, **5)** regular handling, **6)** post-partum stress, **7)** potential poor feeding practices, (26), **8)** social isolation when sick animals are placed in a "hospital pen" (27) and **9)** artificial insemination (28). A stressed, immunocompromised dairy animal is susceptible to mycotic infection. The provision of *A. fumigatus*-infected feed to this animal may be a "trigger" which elicits HBS.

Feed-borne *Aspergillus fumigatus* infects the GI tract, tissues and blood of ruminants. Results of *A. fumigatus* genomic analysis from eight HBS cows, one abomasal hemorrhage (AH) cow and one AH gazelle are shown in Table 1. *A. fumigatus* was detected in 3 of 3 (3/3) feeds submitted for analysis. It was also detected in the gut contents (7 of 7 cases), gut wall (5 of 5 cases), and mesenteric lymph node (3 of 5 cases). Invasive aspergillosis was indicated by detection of *A. fumigatus* DNA in blood (6 of 6 cases) and in liver (1 of 1 case). One case of HBS was associated with a late-term abortion. Cotyledons from the case were sampled and also found to harbor *A. fumigatus*. Of interest, *A. fumigatus* DNA was also detected in two cases of abomasal hemorrhage (AH), one in a dairy cow and another in a *Dama* gazelle (Table 1).

Several negative control cows (i.e., non-HBS; n=17) have also been processed and, of these, 14 have tested negative for *A. fumigatus* (data not shown). The remaining cows (n=3) contained very low levels of *A. fumigatus* DNA; near the detection limit of our assay (i.e., < 0.02 X 10^6 *A. fumigatus* genomic units/ml of blood). These levels were 1/20th to 1/50,000th of the levels detected in HBS cows. Two of the negative control cows (which tested negative for *A. fumigatus*) were from cows which had died from unknown causes at two different dairies. Both had exhibited rumen stasis and sudden death but did not have HBS.

Local feeds were tested for the presence of *A. fumigatus*. Feed samples have included mill run, ground corn, grass and corn silages and dried grass hay. Many were infected with *A. fumigatus*. Infection is not always visible (*A. fumigatus* on moist feed (e.g., corn) is dark blue-green). Hence, exposure to *A. fumigatus* may be common. An additional factor, in tandem with *A. fumigatus*, (possibly immunosuppression) predisposes dairy cattle to HBS.

Clostridial toxins. Genotype analysis of five clostridial toxins indicated no correlation between HBS and toxin genes (Table 2). In HBS and AH cases, genes encoding toxins a and e were detected in 3 of 9 analyses. Toxin genes ? and enterotoxin were not detected in any samples. The a and e toxin genes were detected in blood (1 of 3 analyses), jejunal or abomasal clot (2 of 4 analyses) and GI wall (1 of 5 analyses). In negative control cows, the ß toxin gene was detected in blood of 3 of 17 animals (Table 2). Of interest, two of the three negative control cows which harbored the *Clostridium* ß-toxin gene had aborted.

Study A. *In vivo* assessment. Efficacy of OmniGen-AF was tested on local dairies. To date, OmniGen-AF has been introduced at five dairies in the Pacific-Northwest. Dairies ranged in size from 100 to 950 cows. Each had experienced HBS, mycotic abortions, or both. While definitive conclusions from these studies cannot be drawn (because studies on farms are not "controlled" and because dairy numbers remains low) incidence of HBS and mycotic abortions were eliminated following the introduction of OmniGen-AF on these dairies.

Study B: Infection of steers with *A. fumigatus.* Moldy feed from one HBS-afflicted dairy (WA #1; Table 1) was cultured in large-scale and used to infect rolled corn. Infected corn was used as a culture medium for fungal growth by incubating at 27 ^{o}C for 1-month. *A. fumigatus* was detected in the molded corn product after 1 month of culture. The infected corn was fed to beef steers for 21 days (with and without OmniGen-AF) and then removed from the diet. *A. fumigatus* DNA concentrations were monitored in the blood. Infection of steers occurred rapidly. Within 2 days, *A. fumigatus* levels reached nearly 40 million genomic units/ml in infected steers. Infection with *A. fumigatus* caused rapid clearance of *A. flavus* from the blood (Figure 6). Furthermore, addition of the anti-fungal product enhanced the clearance of *A. fumigatus* from the blood of *A. fumigatus*-infected steers (Figure 7).

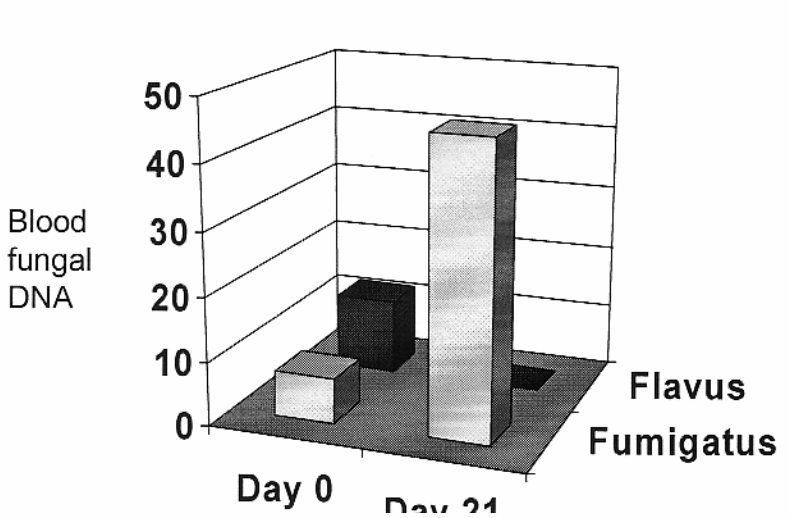

Figure 6. Blood fungal DNA concentrations in beefs steers fed *A. fumigatus*-laden feed for 21 days. Blood was analyzed for *A. fumigatus* and *A. flavus* DNA prior to introduction of feed (Day 0) and after 21 days of feeding. Note that *A. fumigatus* appearance was coincident with clearance of *A. flavus.* Observations are means of two steers/treatment.

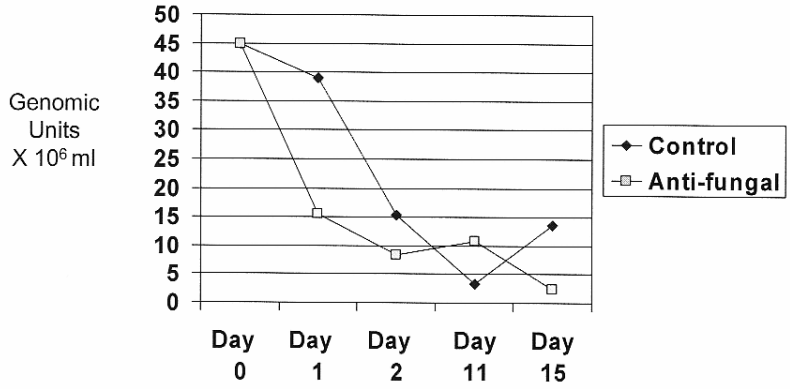

Figure 7. Clearance of *A. fumigatus* from the blood of infected steers. Animals had been fed *A. fumigatus*-infected feed after which feed was withdrawn and clearance of fungal load was monitored over 15 days. The anti-fungal product (OmniGen-AF) enhanced clearance from the blood ($P<0.05$). The experiment included three steers/treatment.

This study demonstrates that HBS is associated with high levels of *A. fumigatus* in the gut, gut wall and blood. In human studies, *A. fumigatus* is recognized for its invasive properties and pathogenicity in immunocompromised patients. We propose it has similar potential in ruminants. Analysis of clostridial toxin genes did not indicate a correlation between these and HBS. Limitations of this analysis include the small data set (i.e., eight HBS cows, two AH animals, 17 controls) and lack of GI tissues from negative control cows. A larger data set and completion of controlled studies with dairy animals are needed to definitively ascribe HBS to *A. fumigatus*. A further limitation is that >100,000 fungal species are known. There are >100 *Aspergillus* species. Only a very small proportion of their ITS domains have been published. Hence, our detection of *A. fumigatus* could include unrecognized (non-sequenced) fungal species. Another issue with HBS is that many cows consume infected feed yet only a small percentage of these cows develop HBS. We do not know what the other predisposing factor(s) may be. However, we propose that stress-induced immunoincompetance may play a role in the etiology of HBS. Finally, we developed and tested a product which inhibits fungal growth *in vitro* and *in vivo.* Application of this product on dairies appears to hold potential for prevention of HBS and, possibly, other mycotic infections.

Limiting Exposure from Improperly Ensiled Forages.

Following best management practices for proper storage and rotation of feed ingredient and ensiled forage inventories can greatly reduce exposure to *Aspergillus fumigatus* growth. Attention to detail when ensiling forages is paramount to the prevention of mold growth. Maturity is important when harvesting forages. Goals for moistures should be 68 to 72% for corn silages and 64 to 68% for legume haylages going into bunker or drive over silos. Silage pits should be continuously and rapidly filled, and immediately packed with a heavy wheeled tractor for best results. Avoid interruptions in

the filling process, if possible, to prevent layers of spoilage from forming. Properly fermented corn silages should reach a pH of 4.0 or less and legume forages will come in below pH 4.5. Innoculants have been beneficial in more rapidly lowering silage pH's. Significant reductions in dry matter losses have been reported with the use of some silage inoculants, however results have historically been variable.

Molds will rapidly grow as the lactic acid from fermentation flashes off from surface layers exposed to the weather. Bunker silos should be immediately covered with quality plastic and weighted down with adequate tires to ensure that the cover will remains firmly in place. Bunker silos should be designed to allow filling from the back, sloping away from the intended active feeding area to prevent rain runoff from draining into exposed feed. The spoiled layer at the top of the silo should be discarded, whenever possible, providing it doesn't pose a safety risk to employees. In a 2003 OSU survey of Pacific Northwest feedstuffs, extremely high concentrations of *Aspergillus fumigatus* spores (>1.25 million per gram) were isolated from the spoiled layer from the top of corn silage bunkers (See Table 3). High levels of A.fumigatus spores were also isolated from separated manure solids used to anchor silo covers (>3 million spores per gram), making this a questionable practice, especially in cases where bunkers are sloped towards the active feeding face.

Compromising hygiene at the feed bunks and water troughs often leads to explosive growth of molds including Aspergillus. Regular cleaning of feed bunks and water troughs should be part of every dairy's HAACP program.

Other recommendations include avoidance of feeding spoiled or molded grains and other feedstuffs to pregnant or lactating animals. Feeding excessively high starch rations can lead to ruminal acidosis and severely damage the gut mucosa, predisposing animals to colonization by invasive molds and other pathogens.

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Table 1. Detection of *A. fumigatus* DNA in samples of blood, tissues and feed recovered from cases of hemorrhagic bowel syndrome and abomasal hemorrhage[1].

Case Location/ID	Diagnosis[2]	Blood	Gut wall	Gut contents	Mesenteric lymph node	Liver	On-site feeds	Cotyledon
WA #1	**HBS**	++	*				+++	
Idaho #1[3]	**HBS**	+++	+++	+	-			
Idaho #2[3]	**HBS**	++++	+++	+++	+			
Iowa Feeds[4]	**HBS**						+++++	
OSUCVM[5]	**AH**		++++	++++		+++		
WA #2	**HBS**		+++++	+++++	++		++	++
Iowa Dairy #1[6]	**HBS**	++++	++	+++	+++++			
Iowa Dairy #2[6]	**HBS**	+++	+++	+++++	-			
Gazelle OR[7]	**AH**		++					
WA #3	**HBS**	+++++						

1-*A. fumigatus* levels were determined and reported as 0 (no detectable *A. fumigatus* DNA) and + to +++++ (low level to very high level). These correspond to *A. fumigatus* loads ranging from 0, 0.2 - 1, 1 - 5, 5 - 20, 20 - 100 and >100 X 10^6 *A. fumigatus* genomic units per g of tissue, per ml of blood or per ml of gut contents (-, +, ++, +++, ++++, and +++++, respectively). **Spaces which are <u>blank</u> in Table 1 indicate that the sample was not provided to us (analyses not completed).**

2-HBS (hemorrhagic bowel syndrome). AH (abomasal hemorrhage). All diagnoses were completed by veterinarians (Oregon State University, University of Idaho), National Animal Disease Center (Iowa State University) or by field veterinarians.

3-Sample was provided by Dr. Bruce Anderson (University of Idaho).

4-Feeds only were obtained from a farm which experienced HBS.

5-Abomasal contents of the AH cows contained high levels of *A. fumigatus*. Gut samples distal to the abomasum were negative. Source: OSUCVM (Dr. Jerry Heidel).

6-Samples were provided by Dr. Mark Rasmussen at the NADC at Iowa State University.

7-A *Dama* gazelle was submitted for necropsy at the OSUCVM. It had been fed in the winter in Oregon and was diagnosed with abomasal hemorrhage. Source: OSUSVM (Dr. Rob Bildfell).
*-DNA sample was extracted; however, sample was not analyzed by omission.

Table 2. Analysis of clostridial toxin genes with multiplex PCR[1]

Case/Location ID	Diagnosis	Blood	Gut contents	Gut wall	Mesenteric lymph node
WA #1	**HBS**	**---**		**---**	
Idaho #1	**HBS**		**A**		**---**
Idaho #2	**HBS**		**---**	**---**	**---**
OSUCVM	**AH**		**---**	**E**	
WA#2	**HBS**			**---**	
Iowa #1	**HBS**	**---**		**---**	**---**
Iowa #2	**HBS**	**a,e**	**A,E**		**---**
Gazelle OR	**AH**			**---**	
WA #3	**HBS**	**---**			
456T	**Negative cow**	**---**			
1046	**Negative cow**	**---**			
606N	**Negative cow**	**---**			
1099N	**Negative cow**	**---**			
S0001	**Negative cow**	**---**			
S0002	**Negative cow**	**---**			
S0005[2]	**Negative cow**	**b**			
S0006[2]	**Negative cow**	**b**			
S0007	**Negative cow**	**---**			
S0020[2]	**Negative cow**	**b**			
S0021	**Negative cow**	**---**			
S0023	**Negative cow**	**---**			
S0024	**Negative cow**	**---**			
S0025	**Negative cow**	**---**			
S0026	**Negative cow**	**---**			
S0027	**Negative cow**	**---**			
S0028	**Negative cow**	**---**			

1- Samples were analyzed for clostridial toxin genes by multiplex PCR. Cells lacking any data were not analyzed. "**---**" indicates non-detectable clostridial toxin genes. "A" and "E" indicate strong a and e signals, respectively. "a", "b" and "e" indicate weak detection of a, ß, and e toxin genes, respectively. Blank cells were not analyzed (usually because they were not submitted for analysis).

2- Cows S0005 and S0006 were from a commercial dairy and had aborted prior to the blood sample. Cow S0020 was a negative control cow in the OSU string.

Table 3. Testing feeds for *A. fumigatus*

Feed	n	*A. fumigatus* positive	% positive	Mean (spores/g)	Range (spores/g)
Corn	5	5	100	11286	675-35775
Corn barley	5	5	100	33156	945-79650
SBM	6	1	16	158	0-945
Beet pulp	9	6	67	28966	0-118125
WCS	10	3	30	15363	0-121500
DDG	10	4	40	11516	0-75625
WMR	5	1	20	6480	0-32400
Spoiled Silage	2	2	100	1300000	1250000-1350000