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Advances in the diagnosis of mycotoxins and endotoxins

AM Craig, LL Blythe, College of Veterinary Medicine, Oregon State University, Corvallis, Oregon, USA

ABSTRACT

Mycotoxins have a long history of causing both animal and human health disease problems. It is estimated that nearly one-quarter of all of the world's food crops are infected with these fungi and their resultant mycotoxins. Aflatoxins, deoxynivalenol (DON), and fumonisins all affect cattle and both the United States and European Union have set regulations for these toxins in feed. Zearalenone, T2-toxin and ochratoxin are also implicated in cattle diseases and performance, but only zearalenone is regulated by the European Union. Analytical methods to screen for these and other toxins are constantly improving as are the reference methods to quantitate each individual toxin. New analyses with liquid chromatography-tandem mass spectrometry can now simultaneously identify up to 186 fungal and bacterial metabolites. However, simple toxin measurement in feed does not take into account ruminal degradation of the toxins, bioavailability in healthy and ill animals as well as the complex diets of both feed lot cattle and dairy cows. As a consequence, the dose of mycotoxins the animal sees systemically and any predictable negative effect on its health remains difficult to ascertain.

INTRODUCTION

Mycotoxins have a long history of either suspected or definitive role in human and animal diseases. From epidemiologic analyses of the ten plagues of Egypt, mycrotoxins have been implicated in the fifth and ten plagues. Plague #5 was labeled animal murrain which was a highly infectious death rate in livestock (Exodus 9:3) and plague #10 was the death of the eldest children which has been hypothesized to be due to molds in the granaries (Exodus 11:4) (Marr and Malloy 1996). In a similar manner, epidemics of ergotism have been recorded since 600 BC with more recent ones occurring in the middle ages in France in 994AD. Earliest documented mycotoxicosis in humans was in tenth century Europe and was referred to as St. Anthony's fire. The disease, ergotism, was caused by the fungus *Claviceps purpurea* infection of grains. Ergotism is also blamed for the outbreak of nervous system disorders in humans in Salem, Massachusetts, USA in 1692 and lead to the execution of many for witchcraft (Meggs 2009). Modern mycotoxicology began in the early 1960s with the outbreak of

the turkey "X" disease in England. The causative agent, aflatoxin, produced by *Aspergillus flavus*, was found in the feeds. Today, the agricultural industry is very aware of many mycotoxins than can affect livestock and regulations have been set in place (CAST 2003). The Food and Agriculture Organization (FAO) has estimated that one quarter of the world's food crops are affected by mycotoxins (Golob 2007).

Toxins as a group can arise from either bacteria, fungi, invertebrates or even vertebrates. Mycotoxins and endotoxins are all secondary metabolites of different fungi that infect plant material either during the growth phase or during storage (Paterson and Lima 2010). The most economically and toxicologically important mycotoxins are the following: aflatoxins, ochratoxins, fumoninsins, deoxynivalenol (DON), zearalenone (ZON), T2-toxins and the ergot alkaloids (Richard 2007). The latter would include both ergot toxins that are evident on the exterior of the plant as dark sclerotia and the endophyte toxins that are not grossly evident and lie within the tissues of certain grasses. Tremorgenic endophytic mycotoxins would include penetrem and lolitrem B.

These toxins find their way into the food chain of both animals and human beings, often with deleterious clinical effects (Fink-Gremmels 2008). These deleterious effects have clinical signs that are unique to the individual toxin and similar to other clinical diseases in animals which makes establishment of a list of differential diagnoses imperative. Definitive diagnosis of mycotoxin toxicity is dependant on chemical identification and quantitation of the respective toxins from the source of infection, *i.*e., the feed being ingested by the animals. While this sounds simplistic, it is often complicated by the fact that dairy cows and finishing beef cows have multiple sources of feed in their diet and it is often high in concentrates with grain being a major component. Dairy cows may have access to pasture grasses, concentrates and silage, all of which can harbor different fungi and different mycotoxins, making multiple analyses of feed material necessary. Sampling techniques, especially those designed to find "hot spots", timing of samplings, gross observation of molds and fungal infection in the exotoxin group are all necessary to establish mycotoxicois as a definitive diagnosis.

Another complicating factor is the fate of the mycotoxins and endotoxins in the rumen. Some mycrotoxins are detoxified by ruminal bacteria or protozoa as seen in Table 1 (Craig and Blythe 1994; Craig 1995). The rate at which this happens depends on the health of the rumen itself, *i.*e., ruminal acidosis might increase or decrease the bioavailability of ingested mycotoxins. Thus the amount of mycotoxins that become systemic is difficult to predict or alter in a ruminant. In fact, the ruminal microbes may even make the ingested mycotoxins more toxic with the production of break-down

metabolities that are equally or even more toxic that the parent molecule. An example would be ergovaline being metabolized to lysergic acid (DeLorme *et al.* 2007).

The first stage of attack to prevent mycotoxicosis would be to know the most likely mycotoxins that infect grains and grasses and measure the mycotoxin levels within them before they become part of the diet. This is especially true of those grains that go into complex concentrate feeds. Tables 2 and 3 list the USA Food and Drug Administration (FDA) and European Union regulations for various mycotoxins.

Analytical measurements of the toxins widely differ between the various toxins. This paper will give an overview of mycotoxins important to cattle industry and discuss current methodologies for analyses.

MYCOTOXINS

Aflatoxins

Aflatoxins have been the most heavily researched of the mycotoxins (Richards 2007). Aflatoxins B1, B2, G1, G2, which are produced from certain strains of the molds *Aspergillus flavus* and *A. parasiticus,* are most abundant in hot, humid geographic areas where corn and cottonseed are grown. Aflatoxin B1 is the most potent. Injury to the growing plant tissue or seed coat can provide a portal of entry of the spores carried by wind or insects. Grains must be stored dry in low moisture/humidity (< 14%), low temperatures (< 20 C), and free from insects to prevent development of increased storage molds.

The effects in animals of ingesting excessive amounts of the toxin range from chronic health and performance problems to death. Aflatoxins are immunosuppressive, carcinogenic, teratogenic, and mutagenic. The target organ within an animal is the liver and the damage results in abnormal blood clotting, development of jaundice, and hemorrhaging. In cattle, reduced feed intake, reduced milk production in dairy cows, and photosensitization may result from ingestion of these mycotoxins. Cockcroft reported tachycardia, tachypnea and death in dairy cows that ingested moldy dairy concentrate that had been lodged in the feed chute (Cockcroft 1995). Ruminal microbes may partially degrade the aflatoxins, but one secondary metabolite is aflatoxicol which is absorbed systemically and hydroxylated by the liver to aflatoxin M which then passes into the urine and the milk. Table 2 lists the FDA and European Community action levels for aflatoxins regulating the levels and species to which contaminated feeds may be fed.

Fumonisins

Fumonisin is a non-fluorescent mycotoxin produced by *Fusarium* sp. and is more commonly found in the southern United States, compared to DON, zearalenone, and T-2 which are prevalent in the northern states. Corn is the major grain affected although some has been found in sorghum and rice. Infection may be grossly visually identified in some corn kernels or areas on the ear and is commonly called "pink kernel rot" although some kernels may be infected with no visual indication. Kernels with insect or bird damage may have high levels of mycotoxins. While increased toxicity during storage does not usually occur, storage conditions should be similar to those described for aflatoxins.

Fumonisin is a proven tumor promoter, a carcinogen, and its effects are most pronounced on horses and monogastrics. Mild liver lesions and decreased milk production can develop in dairy cattle at levels greater than 100 ppm. Usually levels in feeds exist at the 1-10 ppm range. Table 2 lists the FDA and European Community action levels for fumonisin regulating the levels and species to which contaminated feeds may be fed.

T-2 toxin

T-2 toxin is a *Fusarium*-produced mycotoxin and representative of a large group of mycotoxins called trichothecenes (Richard 2007). T-2 toxin has been associated with refusal to eat, lack of weight gain, digestive disorders, intestinal hemorrhages, and death. While data with cattle are limited, the effects of T-2 toxin in laboratory animals are well documented. T-2 toxin is known to suppress immunity and interfere with protein synthesis. It is toxic to the intestine, lymphoid tissues, liver, kidney, spleen, and bone marrow. A calf given T-2 toxin via a stomach tube developed severe depression, pelvic limb ataxia, knuckling of the rear feet, listlessness, and anorexia (Whitlow and Hagler http://www.admani.com/AllianceDairy/TechBulletins/Mycotoxins%20Concerns%20In%20Dairy%20Cattle.htm). It has been demonstrated that at T-2 toxin is associated with gastroenteritis, intestinal hemorrhages, ruminal ulcers and death in dairy cattle (Whitlow and Hagler 2007).

Regulatory and advisory levels for T-2 toxin in the USA are not established but a practical recommendation is to avoid more than 100 ppb of T-2 toxin in the diet.

Deoxynivalenol (DON)

Deoxynivalenol (DON) is the proper name for the most often detected *Fusarium* mycotoxin, often referred to as vomitoxin. Specific modes of action have been identified in swine that explains this toxin as the primary cause of feed refusals, diarrhea, vomiting, reproductive failures, and death. However, DON in cattle has only been associated with reduced feed intake and lower milk production. Whitlow

and colleagues from North Carolina collected clinical data from 300 herds representing about 40,000 cow records showing that DON was associated with a loss in milk production, but did not establish a cause and effect mode of action (Whitlow *et al* 1994). DON may simply be a marker for the presence of other mycotoxins in problem feeds.

The USA's FDA has set advisory levels for DON in grains and grain by-products for cattle older than 4 months at 10 μg/g (not to exceed 50% of diet). The European community has set more stringent regulations.

Ochratoxin A

Ochratoxin A is produced by *Aspergillus sp.* (*A. ochraceus*) and *Penicillium sp.* (*P. viridicatum*). The highest levels are usually found in cereal grains such as corn, barley, wheat and rye. At least nine ochratoxins identified, but ochratoxin A is the most common and has the greatest toxicological significance. It is primarily a kidney toxin but can cause liver damage as well. Ochratoxin A is degraded rapidly in the rumen and would only be a problem in pre-ruminant calves (Whitlow and Hagler 2007).

No FDA action, advisory or guidance levels have been established for ochratoxin A in USA feed.

Zearalenone

Zearalenone is a *Fusarium* produced mycotoxin with a chemical structure similar to estrogen and exposure can produce an estrogenic response in animals. This toxin is commonly found in corn, but can also be found in wheat, barley, occasionally oats and production is favored by high humidity and low temperatures. Zearalenone is often associated with DON but at much lower levels. Ruminants are able to ruminally degrade zearalenone and, therefore, it is less toxic to dairy cattle. Zearalenone may give rise to abortions in dairy cattle, as well as reducing feed intake, milk production and cause vaginitis, vaginal secretions, poor reproduction performance and mammary gland enlargement in virgin heifers. Field observations of poor intakes, depressed milk production, and reproductive problems have been associated with the presence of DON and zearalenone (Whitlow and Hagler 2007).

No FDA action, advisory or guidance levels are established for zearalenone in USA feed but are designated by the European Union (Table 3).

Ergot alkaloids

Ergot alkaloids are toxins produced in two different ways. The major ergot fungus is *Claviceps purpurea* which produces fungal masses in developing seeds which solidify into "ergots" or sclerotia. These are black to purple in color and grossly evident on examination. *C. fusiformis* and *C paspali* are less common with the latter being associated with the paspalitrems that cause dallis grass poisonings (Richard 2007). The major toxin of ergot is ergotamine which causes vasoconstriction of blood vessels resulting in gangrene of the appendages, ears and tails in animals with additional neurological signs in humans. There are no regulatory actions for ergot in grain, but USDA classifies grain containing more that 0.05% sclerotia as "ergoty".

The second way ergot alkaloids are produced is through endophytic fungi that live within the tissue of the plant, in particular, grasses (Blythe *et al* 2007[a]). In cattle in the United States, most exposure is due to ingestion of tall fescue grasses or straw residues that find their way into the diet (Craig 2009). These plants contain the endophyte, *Neotyphodium coenophialum*, which produces ergovaline as the major vasoconstrictive alkaloid. Other ergot alkaloids are often present, but their exact effect on the toxicosis in animals remains poorly defined (Lehner *et al.* 2004; Lehner *et al.* 2005). In warm humid areas such as Southeastern United States, "summer slump" and reproductive problems are the clinical manifestations of tall fescue toxicosis. In cold regions during the winter, "fescue foot' is the fatal disease caused by ergovaline with loss of lower limbs, ears and tails. Ergovaline levels below 350 ppb in cattle are believed to be safe as long as the animals are not in unprotected and prolonged extreme cold temperatures (Tor-Abidye *et al* 2001). Chromatographic methods such as HPLC and LS/MS/MS are used to measure ergot and loline alkaloids quantities in feed material.

Tremorgenic endophytic mycotoxins

There is a group of tremorgenic indole-diterpenes produced by filamentous fungi that have varying detrimental effects on the nervous system of mammals (Saikia *et al.* 2008). Included in this group are the lolitrems, aflatrem, janthitrems B and C, paspalinine, paspalitrems A and B paxilline, penitrems A-F, and terpendole C. The most common toxic tremorgens are the lolitrem B and other lolitrems; they are found in perennial ryegrasses that are infected with the endophyte *Neotyphodium lolli*. When ingested by cattle at levels with lolitrem B at and above 1800ppb to 2000ppb, clinical signs of "ryegrass staggers" are evident (Blythe *et al* 2007[b]). Lolitrem B is believed to be the toxin most responsible for the staggers and is the molecule that is measured in grass, straw or pellets to determine the diagnosis. Lolitrem B has been shown to have its neuromuscular effects by transiently blocking calcium activated potassium channels in skeletal muscle. (Imlach *et al.* 2008).

ANALYSES OF MYCOTOXINS

Sampling

"In many cases, the error induced by the sampling operation is much greater than what occurs at the analysis step." (Blanc 2006). Fungal contamination develops in pockets or "hot spots" in the stored commodity and will not be evenly distributed throughout the lot. Therefore, certain steps need to be taken to reduce sampling error. Gross examination of the sample for presence of fungus should be done initially in the laboratory. Then all samples must be ground to required particle size and blended for homogeneity. Details for handling different types of samples, *i.*e., solid versus liquid, can be found in the publication by Blanc, 2006.

Analytical methods for determining levels of mycotoxins

There are multiple methods for analysis of individual and collections of mycotoxins (Koppen *et al.* 2010). They can be subdivided into screening methods or reference methods.

Rapid screening methods

Mycotoxin immunoaffinity columns: Mycotoxin immunoaffinity columns provide a rapid separation of specific mycotoxins by retaining everything but the mycotoxins of interest. These mycotoxin are then quantified using a fluorometer (Maragos and Busman 2010)

Enzyme-Linked ImmunoSorbent Assay (ELISA): ELISA is an antibody-based assay that is commonly used to detect mycotoxins. A number of commercial ELISA kits are available for aflatoxins, deoxynivalenol, fumonisins, ochratoxins, and zearalenone. This is usually a competitive assay in which the mycotoxin of interest from a sample competes with a labeled mycotoxin for a limited number of specific antibody-binding sites. Since the assay is competitive, the presence of the toxin is usually measured by the absence of color. ELISA is one of the more affordable methods for detecting mycotoxins, but the detection limit often exceeds 0.2 ppm for many mycotoxins (Coffey and Vincelli 2008).

Reference methods

Thin-layer chromatography: Thin-layer chromatography (TLC) is often used as a screening assay. The extracted mycotoxins are spotted on a glass plate which is coated with a thin layer of silica gel. Standard mycotoxins are also applied to the plate and the plate is placed in a development solvent. The

solvent is adsorbed and travels up the plate carrying the spotted standards and extracts. The distance traveled by the compounds from the point of application is influenced by the characteristics of the compound and is measured. The compounds or "spots" are visualized using UV or fluorescent light or sprays (Turner *et al.* 2009).

Gas chromatography: Gas chromatography (GC) is used in the detection and quantification of the trichothecenes which are not amenable to HPLC analysis. The compounds are separated using the relative affinity of the compound for a stationary column and a mobile, inert gas. The separated compounds are often detected using a mass spectrometer (Turner *et al.* 2009).

High-performance liquid chromatography: High-performance liquid chromatography (HPLC) is the most widely used method of mycotoxin analysis. A combination of the immunoaffinity cartridge and HPLC analysis provides sensitive and precise determination of mycotoxins in samples. The extracted mycotoxins are separated using the relative affinity of the compound for a stationary column media and a mobile solvent. The mycotoxins are then quantified using an ultraviolet or fluorescence detector (Turner *et al.* 2009).

Liquid chromatography-mass spectrometry: Liquid chromatography-mass spectrometry (LC-MS) has advanced the detection of the mycotoxins which produce little ultraviolet/visible absorbance or fluorescence. This technique also provides multiple mycotoxin and metabolite detection using a single sample preparation (Nielsèn and Smedsgaard 2003; Lehner *et al.* 2004; Lehner *et al.* 2005; Sorensen and Elbaek 2005; Vishwanath *et al.* 2009). In Vishwanath *et al.* (2009), 186 fungal and bacterial metabolites were simultaneously identified.

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