Mycotoxins in Fungal Contaminated Samples of Animal Feed from Western Canada, 1982–1994

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ABSTRACT

Feed samples from 94 cases involving fungal contamination and suspected mycotoxicosis of farm animals in western Canada were examined during 1982–1994 to assess the incidence of mycotoxins. Samples were analyzed for aflatoxins, ochratoxin A, citrinin, sterigmatocystin, and the fungal estrogen zearalenone. Samples infected with *Fusarium* fungi were additionally assayed for nivalenol, deoxynivalenol, fusarenone-x, 15-acetyldeoxynivalenol, diacetoxyscirpenol, HT-2 toxin, and T-2 toxin. Mycotoxins were found in 21 feed samples from 17 cases (18% of the reported cases), generally at levels far below those needed to induce symptoms under laboratory conditions. HT-2 toxin and other type-A trichothecenes were detected in 5 samples, deoxynivalenol and other type-B trichothecenes in 13, ochratoxin A in 5, and citrinin in 2. In 9 cases, symptoms observed in the animals were consistent with the known effects of the mycotoxin(s) found in the particular feed samples.

RÉSUMÉ

Dans l'ouest canadien, entre 1982 et 1994, des aliments prélevés lors de 94 cas de contamination par des fungus et supectés d'avoir causé des mycotoxicoses ont été analysés pour la recherche d'aflatoxine, d'ochratoxine A, de citrinine, de stérigmatocystine et de zéaralénone. Les échantillons contaminés par *Fusarium* ont de plus été analysés pour détecter la présence de nivalénol, de déoxynivalénol, de fusarénone-x, de 15-acétyldéoxynivalénol, de diacétoxyscirpénol et des toxines T-2 et HT-2. Des mycotoxines ont été retrouvées dans 21 échantillons (17 cas) à des niveaux inférieurs à ceux induisant des signes cliniques dans des conditions de laboratoire. La toxine HT-2 et autres trichotécènes de type A furent détectées dans 5 échantillons, le déoxynivalénol et autres trichothécènes de type B dans 13 échantillons, l'ochratoxine et la citrine dans respectivement 5 et 2 échantillons. Dans 9 cas, les signes cliniques observés concordaient avec les effets connus des mycotoxines retrouvées dans les aliments.

(Traduit par docteur Pascal Dubreuil)

INTRODUCTION

Mycotoxins are fungal metabolites toxic to humans and animals. Mycotoxin development in agricultural crops can occur at various times — during preharvest growth, during harvesting and drying, and during storage (1). Contamination is an additive process. For example, mycotoxin contamination can start with production by *Fusarium* species in the field, and build up during the harvesting and drying operations; additional toxins can be produced in storage due to the action of storage molds, mainly *Penicillium* and *Aspergillus* species. Mycotoxin contamination of feed ingredients affects the agricultural economy not only through diminished animal performance, but also through problems with contaminated animal products.

Research efforts have identified several mycotoxins present in western Canadian feedstuffs, animal tissues, and animal blood. In 1976, Puls and Greenway (2) observed T-2 toxin in samples of barley associated with mycotoxicosis in geese, ducks, horses and swine. Later, Prior (3,4) found aflatoxins, ochratoxin A, and *Fusarium* trichothecenes in approx 4% of 655 feed and tissue samples collected at random. In 1983, Abramson et al (5) analyzed feed samples mostly associated with health problems in chickens and cattle, and detected ochratoxin A, sterigmatocystin, and trichothecenes in 10% of the 51 cases. In 1988, Marquardt et al (6) assayed ochratoxin A in the serum of slaughter hogs, and found levels > 10 ng/mL in 7.25% of 1200 animals.

The foregoing data suggest that most mycotoxin problems posed to western Canadian animal producers arise from a limited number of these compounds. Aflatoxins in feed samples are likely to originate from imported feed ingredients, since trials with Manitoba isolates of *Aspergillus flavus* Link produced no aflatoxins on cereal and oilseed substrates (7). In contrast, the production in western Canada of ochratoxin A and citrinin by *Penicillium* species and sterigmatocystin by *Aspergillus versicolor* (Vuill.) Tiraboschi on feed ingredients during storage are well documented (8,9,10,11). *Fusarium* species and trichothecene mycotoxins have spread through cereal crops in southern Manitoba since 1984 (12); these fungi have been encountered in southern Alberta as well. Grains infected by some species of *Fusarium*, such as *F. graminearum* (Schwabe) and *F. culmorum* (W.G. Smith) commonly contain deoxynivalenol (DON) (12) and other structurally-related trichothecenes (Fig. 1).

The aims of this study were to determine the incidence of mycotoxins in feed samples from western Canada during 1982 and 1994 involving fungal contamination and suspected mycotoxicosis of farm animals, and to

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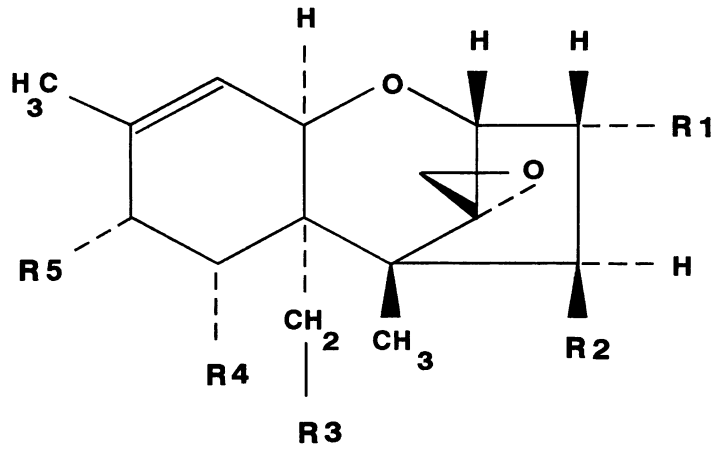

	R1	R2	R3	R4	R5
Deoxynivalenol	OH	H	OH	OH	=O
15-Acetyldeoxynivalenol	OH	H	Acetyl	OH	=O
Nivalenol	OH	OH	OH	OH	=O
T-2 toxin	OH	Acetyl	Acetyl	H	Isopentenyl
HT-2 toxin	OH	OH	Acetyl	H	Isopentenyl
Diacetoxyscirpenol	OH	Acetyl	Acetyl	H	H

Figure 1. Structures of trichothecene mycotoxins detected in western Canadian animal feed samples.

ascertain which mycotoxins to include in future screening procedures.

MATERIALS AND METHODS

STANDARDS

Mycotoxin and fungal estrogen standards were obtained from Sigma Chemical Co. (St. Louis, Missouri, USA), except for fusarenone-X and nivalenol (Wako Pure Chemical Industries, Richmond, Virginia, USA). DON was a gift from Dr. Roy Greenhalgh, formerly of Plant Research Center, Agriculture and Agri-Food Canada, Ottawa, Ontario. Isobenzan ("Telodrin") internal standard for gas chromatography/mass spectrometry (GC/MS) was obtained from Chromatographic Specialties Ltd., Brockville, Ontario.

SAMPLING

One hundred and six feed samples of approx 400 g were received from farmers, veterinarians, or feed mill operators from 94 cases where feeds or feed ingredients were suspected to contain mycotoxins. In 85 cases the complaints arose due to symptoms in animals consuming the feed; in 9 cases, concern arose due to the moldy deteriorated appearance of the feed. Samples were received from British Columbia, Alberta, Saskatchewan, and Manitoba between May 1982 and May 1994. At time of submission, a brief written description was requested describing any animal health problems associated with the feed sample(s). The reported problems were examined for possible relevance to mycotoxins found in the feed samples.

DETECTION OF *Fusarium* SPECIES

Although *Fusarium* species on grain samples are viable for months at 20–30°C (13) and for years at −20°C (14), samples were assayed for these fungi immediately upon receipt (usually within a month of the complaint), and stored at −20°C afterwards. *Fusarium* species were detected by placing 50 seeds onto potato dextrose agar. Samples were plated without surface sterilization, 10 seeds per dish, and incubated for 7 d at 22°C under alternating fluorescent and longwave ultraviolet light, 12 h of each. Commercial feeds were ground in a centrifugal mill with 0.5-mm screen apertures, and 50–100 mg ground material was placed at 10 locations on the agar surface. Fungal identification was confirmed by light microscopy (15).

SCREENING FOR MYCOTOXINS

Samples were ground to pass through an 0.85 mm aperture screen, and 50 g of ground material were shaken with 25 mL of 0.5 N phosphoric acid and 250 mL of chloroform for 30 min. During filtration through Whatman 308 paper, two 50 mL fractions were collected. The 1st was processed by gel filtration (16), and the purified residues were assayed for aflatoxins B_1, B_2, G_1, and G_2, sterigmatocystin and zearalenone by thin-layer chromatography (TLC) on silica plates. The 2nd was purified using acidic celite (17), and similarly assayed for ochratoxin A and citrinin. Positive samples were then quantitated by liquid chromatography. Limits of detection for aflatoxin B_1 and ochratoxin A were 2 µg/kg, for citrinin 10 µg/kg, and for sterigmatocystin and zearalenone 50 µg/kg.

Samples which were positive by silica TLC were confirmed against standards by TLC on reverse-phase C_{18}-bonded plates to eliminate false positives (18). Confirmation of ochratoxin A involved the preparation of the methyl ester (19). Confirmation of sterigmatocystin involved the preparation of the acetylation product (20).

QUANTITATION OF MYCOTOXINS

Quantitative estimation of ochratoxin A and citrinin was performed by liquid chromatography using a C_{18} bonded-phase adsorbant. Ochratoxin A was eluted using 35% methanol and 65% aqueous formic acid (2% formic acid with 98% water), and detected by fluorescence at 365 nm irradiation and > 418 nm emission. Citrinin was eluted with 50% methanol and 50% aqueous formic acid, and detected in the same manner.

Because of the complexity of GC/MS analysis, samples were assayed for trichothecenes only if *Fusarium* species were found. Fifty gram portions of these samples were

ground and extracted, and the extracts treated with ammonium sulfate and partitioned (21). The extracts were further purified using silica columns as described, and the purified residues converted to the heptafluorobutyryl esters with HFBI. The esters were mixed with isobenzan (internal standard) and injected into a Hewlett-Packard 5985 quadrupole GC/MS unit, separated using a 20 m long 0.20 mm diameter column coated with phenylmethyl silicone, and detected by electron-impact ionization. Samples were quantitated and confirmed for trichothecenes by monitoring the following ions: nivalenol, 321 and 283; DON, 332 and 359; fusarenone-x, 669 and 627; 15-ADON, 444 and 321; diacetoxyscirpenol, 502 and 429; HT-2 toxin, 179 and 205; and T-2 toxin, 179 and 205. Limits of detection were 50 µg/kg for nivalenol, 10 µg/kg for diacetoxyscirpenol and T-2 toxin, and 1 µg/kg for the other trichothecenes.

RESULTS AND DISCUSSION

Features of the suspect feed samples are summarized in TABLE 1. Major problems were encountered trying to obtain representative samples when collecting feed from farms. In almost all cases, symptoms were not noted, and samples were not collected, until most of the feed was consumed. Generally, there was no employment of special sampling equipment or of formal sampling techniques, such as the cone-and-quarter method. In stored grain, mycotoxins often occur in distinct zones in the storage structure where moisture has accumulated; subsequent remixing of the grain during transport or feeding creates a heterogeneous mycotoxin distribution and problems in representative sampling. In some cases (822,833,838) multiple feed samples from the same source were submitted.

Of the 94 cases investigated, animal health problems were involved in 85, and mycotoxins were detected in 17. In 13 of these cases, feed samples were associated with complaints about livestock health and productivity. In the other 4 cases, samples had been sent in as a cautionary measure, but the grain was not fed to livestock.

In 13 cases feed samples were mycotoxin-positive (TABLE 1), and health problems had been documented in brief reports accompanying the samples. Symptoms in the affected animals corresponding to some of the properties of the mycotoxins (review: reference 22) were noted in 9 instances. In case 507, presence of wild bird carcasses near the feed was consistent with accounts of high mortality due to ochratoxin A and citrinin; the toxic activity of this mycotoxin combination in poultry has been described (23,24). In case 513, loss of milk production in dairy cattle agreed with the known effects of ochratoxin A. In cases 520, 820, 821, and 838, feed refusal was reported in swine, consistent with a known property of DON. In case 803, reduced growth rate was observed in the poultry, corresponding to the known effects of long-term low ochratoxin levels. In case 809, gastro-intestinal hemorrhagic symptoms were noted in beef cattle; these effects have been reported in cattle for some of the more toxic type-A trichothecenes, mainly T-2 toxin, HT-2 toxin, and diacetoxyscirpenol. In case 833, high mortality was observed in a poultry flock, and this is consistent with accounts of ochratoxin poisoning described in the literature (23).

Except for 2 cases (507 and 833), mycotoxin levels were far below those needed to induce symptoms in laboratory studies. This discrepancy may be due in part to non-representative sampling of the feed, to low recovery of mycotoxins from the feed during extraction, and to other factors. Hamilton and co-workers (25) have commented on the difficulty of correlating mycotoxin levels in suspect feed and severity of observed symptoms, and of relating this correlation to dose-response curves developed in the laboratory. Such a correlation would require representative sampling, uniform distribution of the

TABLE I. Features of mycotoxin-positive feed samples collected in western Canada during 1982–1994

Case #	Feed	Animals consuming feed	Mycotoxin[a]	µg/kg Feed
3	barley	dairy cattle	citrinin	10
507	wheat	wild birds	citrinin	4400
			ochratoxin A	1100
513	barley	dairy cattle	ochratoxin A	60
520	wheat+barley	swine	DON	95
525	barley	[b]	DON	70
			HT-2 toxin	3
			T-2 toxin	14
526	corn	[b]	DON	20
			DAS	19
			HT-2 toxin	1
803	commercial mix	poultry	ochratoxin A	5
808	corn	[b]	DON	105
			15ADON	80
809	mixed grains	beef cattle	HT-2 toxin	66
810	commercial mix	swine	DON	19
811	commercial mix	poultry	DON	19
820	commercial mix	swine	DON	45
			15ADON	11
821	barley	swine	DON	16
822	barley	swine	HT-2 toxin	53
822	corn	swine	nivalenol	311
			DON	33
			15ADON	16
825	barley	[b]	HT-2 toxin	41
833	wheat	poultry	DON	200
			ochratoxin A	900
833	mixed grains	poultry	ochratoxin A	1600
838	wheat	swine	DON	20
838	barley	swine	DON	22
			15ADON	5
838	barley	swine	nivalenol	65
			DON	13

[a] DAS=diacetoxyscirpenol; DON=deoxynivalenol; 15ADON=15-acetyldeoxynivalenol
[b] not fed to livestock because of moldy appearance

toxin throughout the feed, identical environments, no interaction among the toxin(s) and other factors, no changes in diet or management, complete characterization of the toxicosis, and same genotypes in the different episodes. Protocols for representative sampling of commodities for mycotoxin analysis have been suggested by various testing and standards organizations (26).

Of the 21 mycotoxin-positive samples of suspect feed in TABLE 1, HT-2 toxin and other type-A trichothecenes were present in 5, DON and other type-B trichothecenes in 13, ochratoxin A in 5, and citrinin in 2. The increase in DON incidence, compared to surveys from the 1970's and early 1980's (3,4,5), reflects both the improvement in analysis methods, and the spread of fusarium head blight in cereal crops. Feed handlers, animal health personnel, and testing laboratories should be alert to the possibility of type-A and type-B trichothecenes, ochratoxin A, and citrinin in feed samples, and include these toxins in future screening protocols. Analytical kits based on the enzyme immunoassay principle (27) are available for most of these mycotoxins; commercial immunoassays for citrinin (28) may be available in the future.

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