Fumonisin Concentrations in Brazilian Feeds Associated with Field Outbreaks of Confirmed and Suspected Animal Mycotoxicoses

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Twenty-one *Fusarium moniliforme*-contaminated feed samples associated with outbreaks of confirmed and suspected mycotoxicoses in various animal species were collected from farms in the State of Paraná, Brazil, and analyzed for the fumonisins. Fumonisins B_1 (FB_1) and B_2 (FB_2) were detected in 20 and 18 of the 21 feed samples, respectively, at concentrations of 0.2–38.5 $\mu g\ g^{-1}$ FB_1 and 0.1–12.0 $\mu g\ g^{-1}$ FB_2. In addition, duckling toxicity and fumonisin levels were determined in corn cultures of 26 *F. moniliforme* isolates from the feed samples. With the exception of one isolate, all were acutely toxic to ducklings and contained 65–4420 $\mu g\ g^{-1}$ FB_1 and 5–1380 $\mu g\ g^{-1}$ FB_2. The results constitute the first report on the natural occurrence of the fumonisins in animal feeds from Brazil.

INTRODUCTION

Fusarium moniliforme Sheldon, a worldwide contaminant of corn, has been associated with diseases in both animals and man (Marasas et al., 1984). Possibly the most widely reported animal disease syndrome associated with the ingestion of *F. moniliforme*-contaminated feed is equine leukoencephalomalacia (LEM). The characteristic clinical signs and pathological changes of LEM in horses and donkeys include apathy, nervous disorders, paralysis of the lower lip, mobility problems, and liquefactive necrosis of the white matter of the cerebral hemispheres (Marasas et al., 1984; Kellerman et al., 1988). Field outbreaks of LEM have occurred sporadically in many countries including Argentina, Brazil, China, Egypt, South Africa, and the United States (Marasas et al., 1984). As recently as 1989, widespread outbreaks of equine LEM were reported in the United States (Ross et al., 1990; Wilson et al., 1990a,b).

Disease syndromes associated with the ingestion of *F. moniliforme*-contaminated feed are not, however, restricted to members of the Equidae family. Kriek et al. (1981) reported the death of two of three pigs fed a diet containing culture material of *F. moniliforme*, where the principal lesions were pulmonary edema (PE). Recently, Harrison et al. (1990) observed indentical lesions in addition to hydrothorax in pigs, following the deaths of 34 swine fed a diet containing *F. moniliforme*-contaminated corn screenings of the 1989 U.S. crop.

Gelderblom et al. (1988) reported the isolation of a group of structurally related secondary metabolites, the fumonisins, from culture material of *F. moniliforme* strain MRC 826, which had been shown to cause equine LEM (Kriek et al., 1981). To date, two fumonisin A and four fumonisin B mycotoxins have been isolated and characterized (Bezuidenhout et al., 1989; Gelderblom et al., 1988; Cawood et al., 1991). Cultures of *F. proliferatum* (Matsushima) Nirenberg and *F. nygamai* Burgess & Trimboli have also been shown to produce the fumonisins (Ross et al., 1990; Thiel et al., 1991a). Toxicological studies have resulted in the experimental reproduction of both equine LEM (Marasas et al., 1988; Kellerman et al., 1990) and porcine PE (Harrison et al., 1990) following administration of pure FB_1, and FB_1 has also been shown to be both hepatotoxic and carcinogenic to rats (Gelderblom et al., 1991a). In addition to the major fumonisin (FB_1), two others, fumonisin B_2 (FB_2) and fumonisin B_3 (FB_3), are also produced in significant quantities by cultures of *F. moniliforme* (Cawood et al., 1991). Recent studies have shown FB_2 and FB_3 to possess toxic and cancer-initiating properties similar to those observed for FB_1 (Gelderblom et al., 1991b).

Assessment of fumonisin levels in naturally contaminated foods and feedstuffs, in conjunction with data on the toxic and carcinogenic potential of the fumonisins in experimental animals, will be utilized to set tolerance levels and legislative regulations, on a scientific base, to reduce the risk of fumonisin exposure to both animals and man. Methods for the analytical determination of the fumonisins in human foodstuffs and animal feeds have been published (Gelderblom et al., 1988; Plattner et al., 1990; Shephard et al., 1990; Sydenham et al., 1990; Wilson et al., 1990b), and some have been used to determine fumonisin levels in feed samples associated with outbreaks of either equine LEM or porcine PE (Harrison et al., 1990; Plattner et al., 1990; Shephard et al., 1990; Ross et al., 1990; Wilson et al., 1990b; Thiel et al., 1991b). The high-performance liquid chromatographic (HPLC) procedure for the determination of FB_1 and FB_2 in corn-based foods and mixed feeds, first described by Shephard et al. (1990), has been the subject of a recently completed international collaborative study (Thiel et al., 1991c). This method was used to analyze feed samples collected between mid 1985 and the latter part of 1990 from field outbreaks of confirmed and suspected mycotoxicoses, in several different animal species in the State of Paraná, Brazil. This paper reports the results of those analyses and, in addition, documents fumonisin production and duckling toxicity results of *F. moniliforme* isolates from the Brazilian feed samples.

EXPERIMENTAL PROCEDURES

Feed Samples. Over a 5-year period, between July 1985 and October 1990, 21 corn or corn-based mixed feed samples, associated with outbreaks of confirmed and suspected animal

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Table I. Feed Samples Associated with Outbreaks of Mycotoxicoses in Different Animal Species in Brazil[a]

sample	feed composition	animals affected	disease syndrome/clinical signs and no. of deaths[b]
1	mixed ground corn	horses	LEM[c] (7 deaths)
2	corn residue	horses	LEM (2 deaths)
3	corn residue	horses	LEM (1 death)
4	mixed corn	pigs	PE[d] (60 deaths)
5	corn	piglets	elevated temperature (15 deaths)
6	corn	rabbits	nervous/feed refusal (5 deaths)
7	corn	horses	early LEM (no deaths)
8	corn	pigs	estrogenic effects (no deaths)
9	chicken feed	chickens	diarrhea (no deaths)
10	corn	pigs	PE (no information on deaths)
11	ground corn and straw	horses	LEM (1 death)
12	mixed feed	horses	LEM (1 death)
13	corn residue	pigs	PE (1 death)
14	corn ears	horses	LEM (no information on deaths)
15	black oats	horses	LEM (1 death)
16	ground corn and straw	horses	samples were from the same farm, LEM (3 deaths)
17	ground corn and straw	horses	
18	ground corn	horses	
19	ground corn	horses	
20	ground corn	horses	
21	ground corn	horses	early LEM (no deaths)

[a] Feed samples collected from farms in the State of Paraná, Brazil, between July 1985 and October 1990. [b] Information given where available. [c] LEM, leukoencephalomalacia. [d] PE, pulmonary edema.

mycotoxicoses, were collected from farms in the State of Paraná, Brazil (Table I).

Fungal Cultures. Fungi were isolated and enumerated from the 21 feed samples by means of dilution plating (Nelson et al., 1983) on coconut agar (Lin and Diannese, 1976) containing 0.3% Triton X-100 and 50 μg mL^{-1} chloramphenicol. Plates were incubated at 25 °C and counts made after 7 days. Selected *Fusarium* cultures that developed from each feed sample were transferred to potato dextrose agar (PDA) and carnation leaf agar (Fischer et al., 1982) and identified according to the method of Nelson et al. (1983). One or more representative isolates of *F. moniliforme* from each feed sample were single-spored, lyophilized, and deposited in the culture collection of the Medical Research Council (MRC), Tygerberg, South Africa. Lyophilized conidia of 26 isolates of *F. moniliforme* from the 21 feed samples were used to inoculate autoclaved corn and tested for toxicity to 1-day-old Pekin ducklings as previously described (Marasas et al., 1979; Jeschke et al., 1987; Wilson et al., 1990).

Fumonisin Determinations. Prior to analyses, feed and culture samples were ground to a fine meal in a laboratory mill. Subsamples were analyzed for the presence of FB_1 and FB_2 according to the method described by Shephard et al. (1990). Briefly, subsamples were extracted with a 3:1 mixture of methanol/water. Following extraction and filtration, aliquots of the crude extracts were applied to preconditioned strong anion exchange (SAX) cartridges. The fumonisins were eluted from the cartridges with a 1% solution of acetic acid in methanol, the eluent being collected and evaporated to dryness and the residue redissolved in methanol. Following derivatization with *o*-phthaldialdehyde (OPA), derivatized extracts were separated by reversed-phase HPLC utilizing fluorescence detection. Quantification was by comparison of toxin peak areas observed in the sample extracts, with those obtained for similarly derivatized authentic standards.

Mycotoxin Standards. Fumonisin analytical standards were isolated from culture material of *F. moniliforme* MRC 826 according to the procedure published by Gelderblom et al. (1988). The identities of the toxins were confirmed by nuclear magnetic resonance spectroscopy. Thin-layer chromatographic and HPLC analyses of the standards indicated the presence of a single component per standard.

Table II. Total Fungal Counts, *Fusarium* Counts, and Fumonisin Concentrations in Brazilian Feed Samples Associated with Outbreaks of Mycotoxicoses in Different Animal Species

	propagules/g[a]		fumonisin concn, μg g^{-1}	
sample	total	*Fusarium* spp.[b]	FB_1	FB_2
1	1.6×10^7	1.5×10^7	24.2	8.3
2	3.2×10^6	8.0×10^5	38.5	12.0
3	2.8×10^5	1.8×10^5	20.8	6.6
4	1.1×10^6	5.0×10^5	8.5	1.9
5	2.8×10^5	3.0×10^4	0.2	ND[c]
6	2.5×10^6	1.0×10^5	ND	ND
7	1.0×10^4	–[d]	0.6	0.1
8	3.6×10^5	1.2×10^5	7.0	1.5
9	5.1×10^5	4.3×10^5	5.1	1.2
10	6.6×10^6	6.5×10^5	11.1	3.2
11	1.6×10^8	1.6×10^8	2.6	0.7
12	9.0×10^3	–[d]	4.5	0.8
13	3.3×10^6	2.9×10^6	10.4	3.2
14	1.2×10^7	3.9×10^6	19.2	8.0
15	4.8×10^4	3.0×10^4	0.2	0.1
16	2.0×10^8	2.0×10^7	7.4	3.7
17	1.5×10^5	6.0×10^4	0.8	0.2
18	2.4×10^6	2.0×10^6	22.9	7.9
19	3.7×10^6	2.5×10^6	0.9	0.1
20	3.1×10^6	1.0×10^6	1.9	0.3
21	1.1×10^7	9.0×10^6	0.2	ND

[a] Counts on coconut agar plus 0.3% Triton X-100 and 50 μg mL^{-1} chloramphenicol, incubated at 25 °C for 7 days. [b] Mostly *F. moniliforme* and at least one isolate of this species were obtained in pure culture for each feed sample. [c] ND, not detected (<0.05 μg g^{-1}). [d] *Fusarium* counts not done.

RESULTS AND DISCUSSION

The field outbreaks of mycotoxicoses that occurred in the State of Paraná, Brazil, affected several different animal species including horses, pigs, rabbits, and poultry (Table I). The levels of FB_1 and FB_2 determined in the 21 Brazilian feed samples are listed in Table II. All of the feed samples were contaminated with detectable levels of FB_1 and FB_2, with the exception of one sample which contained neither toxin and two other samples which were negative for FB_2. The levels of FB_1 detected in the samples (Table II) were in each case higher than the corresponding FB_2 levels. These results are in accordance with those previously reported for fungal cultures of *F. moniliforme* (Gelderblom et al., 1988; Shephard et al., 1990; Thiel et al., 1991a), for samples associated with confirmed cases of equine LEM (Wilson et al., 1990b; Thiel et al., 1990b), and for commercially available corn-based human foodstuffs (Sydenham et al., 1991b).

Following pathological examinations, equine LEM was diagnosed as the cause of death in 12 of the 21 cases of mycotoxicoses (Table I). The fumonisin levels determined in the 12 implicated feed samples were found to be 0.2–38.5 μg g^{-1} for FB_1 and 0.1–12.0 μg g^{-1} for FB_2, with mean concentrations of 12.0 and 4.1 μg g^{-1}, respectively. These levels were similar to those previously reported for one sample from an outbreak of LEM in South Africa [8.9 μg g^{-1} FB_1 and 3.0 μg g^{-1} FB_2 (Shephard et al., 1990)] and for 14 samples from the United States (Thiel et al., 1991b), where mean concentrations of 7.7 μg g^{-1} FB_1 and 3.1 μg g^{-1} FB_2 were reported. Far higher fumonisin concentrations were recorded in three U.S. samples by Wilson et al. (1990b), where FB_1 levels ranged between 37 and 122 μg g^{-1} (mean of 72 μg g^{-1}) and FB_2 levels between 2 and 23 μg g^{-1} (mean of 12 μg g^{-1}). Ross et al. (1991) recently reported FB_1 contamination of 98 samples associated with 44 cases of equine LEM in several states of the United States, where levels between 1 and 126 μg g^{-1} with a mean

of 24.6 $\mu g\ g^{-1}$ were recorded. Two other Brazilian samples that had been implicated in the early clinical symptoms of equine LEM [samples 7 and 21, Table I] were contaminated with relatively low mean concentrations of FB_1 and FB_2, at 0.4 and 0.05 $\mu g\ g^{-1}$, respectively.

Only 3 of 21 Brazilian feed samples were implicated in confirmed cases of porcine PE (samples 4, 10, and 13, Table I). The mean concentrations determined in these samples were 10.0 $\mu g\ g^{-1}$ FB_1 and 2.8 $\mu g\ g^{-1}$ FB_2. Harrison et al. (1990) reported a mean concentration of 130 $\mu g\ g^{-1}$ FB_1 in two samples associated with outbreaks of porcine PE in the United States, while levels between 1 and 330 $\mu g\ g^{-1}$ FB_1 (mean of 55 $\mu g\ g^{-1}$) were reported for 83 samples associated with 42 cases of porcine PE in the United States (Ross et al., 1991).

In the case of sample 5, which contained a low level of FB_1 (0.2 $\mu g\ g^{-1}$) and no FB_2 (Table II), only piglets under the age of 70 days appeared to be affected. Another sample associated with an outbreak of suspected porcine mycotoxicosis was sample 8, which contained 7.0 $\mu g\ g^{-1}$ FB_1 and 1.5 $\mu g\ g^{-1}$ FB_2. However, no deaths were recorded following consumption of the feed, and the principal clinical signs observed in the pigs appeared to be estrogenic effects (Table I). Such effects have been well correlated with the presence of zearalenone (ZEA) and its derivatives in feeds (Marasas et al., 1984). ZEA is not normally regarded to be a *F. moniliforme* mycotoxin, although there have been some reports suggesting that it is produced at low levels by some *F. moniliforme* isolates (Marasas et al., 1984). Although not classified as mycotoxins, the gibberellins (plant-growth promotors) have been isolated from culture material of *F. moniliforme*, and one of these compounds, gibberellic acid, has been reported to exhibit estrogenic effects (Marasas et al., 1984). Unfortunately, insufficient quantities of sample 8 were available for any additional mycotoxin analyses.

The remaining two Brazilian samples were implicated in outbreaks of suspected mycotoxicoses in rabbits (sample 6) and in chickens (sample 9) (Table I). The clinical signs in the rabbits included nervous symptoms such as loss of equilibrium and paralysis and feed refusal. No fumonisins were detected in the feed sample, nor could reports concerning similar clinical signs in rabbits consuming *F. moniliforme*-contaminated feed be found in the literature. Sample 9 contained 5.1 $\mu g\ g^{-1}$ FB_1 and 1.2 $\mu g\ g^{-1}$ FB_2. The disease that affected the chickens was characterized by diarrhea and hemorrhagic lesions in the gastrointestinal tract. Such a hemorrhagic syndrome may have been caused by trichothecene mycotoxins but has not been attributed to the consumption of *F. moniliforme*-contaminated feed by poultry (Marasas et al., 1984). It must be concluded, therefore, that in the present study the toxic principle responsible for the symptoms observed in the rabbits and chickens is as yet unknown.

The results of the mycological analyses of the feed samples are summarized in Table II. It is clear that *Fusarium* spp., comprising mostly *F. moniliforme*, were the predominant fungal contaminants of most of the feed samples. It was possible to obtain at least one representative isolate of *F. moniliforme* in pure culture from each sample. The duckling toxicity data and fumonisin levels determined for the 26 isolates of *F. moniliforme*, grown on corn, isolated from the 21 Brazilian feed samples are given in Table III. With the exception of MRC 6073, all other *F. moniliforme* cultures were acutely toxic to ducklings. This is in agreement with previous reports on the toxicity to ducklings of *F. moniliforme* isolates from corn and feeds (Marasas et al., 1979; Jeschke et al., 1987; Wilson et al., 1990). Corn cultures of all 26 isolates of *F. moniliforme* contained FB_1 at levels ranging from 65 to 4420 $\mu g\ g^{-1}$ and FB_2 at 5–1380 $\mu g\ g^{-1}$. The lowest levels of both FB_1 and FB_2 occurred in the culture material of MRC 6073, which was nontoxic to ducklings. In accordance with previous papers concerning fungal cultures of *F. moniliforme* (Gelderblom et al., 1988; Ross et al., 1990; Shephard et al., 1990; Thiel et al., 1991a), FB_1 was the major fumonisin produced, corresponding to between 60% and 93% of the combined FB_1 and FB_2 levels determined in the cultures.

Table III. Toxicity to Ducklings and Fumonisin Concentrations of Corn Cultures of 26 Isolates of *F. moniliforme* from Brazilian Feed Samples

		toxicity to ducklings			fumonisin concn, $\mu g\ g^{-1}$	
sample	MRC[a]	mortality, dead/tested	mean days to death	total feed intake,g	FB_1	FB_2
1	6063	4/4	5.3	50	1140	350
2	6064	4/4	5.0	30	1320	430
3	6065	4/4	4.8	32	2260	520
4	6066	4/4	5.3	22	3660	970
5	6073	0/4		1088	65	5
6	6076	4/4	5.0	20	1040	180
7	6078	4/4	4.3	8	530	120
8	6079	4/4	5.0	46	720	210
9	6082	4/4	5.8	18	640	80
10	6084	4/4	5.0	26	1490	980
11	6086	4/4	4.3	22	4420	1380
12	6087	4/4	4.3	16	3650	910
13	6088	4/4	4.3	46	1220	230
14a	6159	4/4	4.5	10	1635	290
14b	6160	4/4	5.0	92	285	100
15	6161	4/4	4.5	16	2280	710
16	6163	4/4	5.0	20	2230	540
17a	6164	4/4	4.5	16	3380	825
17b	6165	4/4	4.3	38	625	200
18a	6166	4/4	5.0	34	910	160
18b	6167	3/4	6.3	478	270	40
19a	6168	4/4	4.5	20	1385	200
19b	6169	4/4	6.0	100	1980	260
20a	6170	4/4	6.0	42	690	65
20b	6171	4/4	4.5	28	1795	290
21	6173	4/4	5.0	12	1570	280

[a] MRC, accession number of *F. moniliforme* isolates in the culture collection of the South African Medical Research Council, Tygerberg, South Africa.

The data presented in Tables II and III do not, however, take into account the presence of a third major fumonisin, FB_3. Gelderblom et al. (1991b) reported that, following short-term carcinogenesis studies in a rat liver bioassay, FB_2 and FB_3 exhibited toxicological and cancer-initiating activities similar to those previously reported for FB_1. In addition, a recent paper indicated FB_3 levels in feed samples associated in field outbreaks of equine LEM in the United States (Sydenham et al., 1991a) corresponding to between 2% and 18% of the total fumonisin content of the feeds, based on the combined $FB_1 + FB_2 + FB_3$ concentrations, in 12 of 13 feed samples tested. During the analyses of the Brazilian samples, it was not possible to quantitatively assess the levels of FB_3, due to the lack of a FB_3 standard of analytical purity. It was possible, however, to qualitatively assess the occurrence of FB_3 in the samples. HPLC peaks corresponding to the chromatographic position of FB_3 were observed in all but 6 of the 21 Brazilian feed samples. The samples that appeared to be negative for FB_3 were those that had the lowest levels of both FB_1 and FB_2 (samples 5–7, 11, 15, and 21, Table II). By contrast, all of the cultured samples exhibited evidence for the presence of FB_3, and in 16 of the 26 cultures the FB_3 levels exceeded their corresponding FB_2 concentrations (assuming that a FB_3-OPA derivative gave a detector response similar to that of the OPA derivative of FB_2). The extent to which FB_2 and FB_3 may contribute

to the development of equine LEM, porcine PE, and other possible animal or human disease syndromes is as yet unknown. However, the evidence of Gelderblom et al. (1991b) on the toxic and carcinogenic potential of FB_2 and FB_3 suggests that they should be considered potentially harmful, and hence their occurrence should be monitored.

In summary, the present data support previous results on the natural occurrence of the fumonisins in feed samples associated with field outbreaks of equine LEM and porcine PE. Although both FB_1 and FB_2 were present in a single feed sample associated with an outbreak of suspected mycotoxicosis in chickens, there is as yet no evidence that the fumonisins were responsible for the observed clinical and pathological effects. In addition, data on the fumonisin-producing ability of *F. moniliforme* isolates from the incriminated samples are presented. This paper is the first report of the natural occurrence of fumonisins in animal feeds from Brazil and of the production of fumonisins by Brazilian isolates of *F. moniliforme* in cultures on corn.

ABBREVIATIONS USED

FB_1, fumonisin B_1; FB_2, fumonisin B_2; FB_3, fumonisin B_3; HPLC, high-performance liquid chromatography; OPA, *o*-phthaldialdehyde; ZEA, zearalenone.

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