Levels of Fumonisins B_1 and B_2 in Feeds Associated with Confirmed Cases of Equine Leukoencephalomalacia

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Leukoencephalomalacia (LEM) is a neurotoxic disease of Equidae caused by the ingestion of feed contaminated with the fungus *Fusarium moniliforme*. Feed samples from the United States that were fed to horses prior to the development of LEM were analyzed for fumonisin B_1 (FB_1) and fumonisin B_2 (FB_2), toxic secondary metabolites of *F. moniliforme*. In addition, FB_1, FB_2, and moniliformin were determined in corn cultures of 10 isolates of *F. moniliforme* from these samples. None of the cultures produced moniliformin but all contained both FB_1 (160–3800 $\mu g\ g^{-1}$) and FB_2 (20–950 $\mu g\ g^{-1}$). All 14 feed samples contained both FB_1 (1.3–27.0 $\mu g\ g^{-1}$) and FB_2 (0.1–12.6 $\mu g\ g^{-1}$). FB_1 was the major fumonisin in the cultures (80–96%) as well as in the feed samples (53–93%). These results support the finding that the fumonisins are causative factors in the development of LEM in horses.

INTRODUCTION

Field outbreaks of leukoencephalomalacia (LEM) occur sporadically in many countries including Argentina, China, Egypt, New Caledonia, Republic of South Africa, and especially the United States of America (Marasas et al., 1984, 1988). This neurotoxic disease of horses and other Equidae is characterized by liquefactive necrosis of the white matter of the cerebral hemispheres (Kellerman et al., 1988). *Fusarium moniliforme* Sheldon, a common contaminant of corn throughout the world, has long been known to be the causative fungus in the development of LEM (Wilson and Maronpot, 1971; Marasas et al., 1976; Kriek et al., 1981). Gelderblom et al. (1988) recently isolated fumonisin B_1 (FB_1) and fumonisin B_2 (FB_2) from a culture of *F. moniliforme* MRC 826 that had been shown to cause LEM in horses (Kriek et al., 1981). The structure of FB_1 was elucidated by Bezuidenhout et al. (1988) and shown to be the diester of propane-1,2,3-tricarboxylic acid and a pentahydroxyicosane containing a primary amino group. FB_2 was shown to be the C-10 deoxy analogue of FB_1.

FB_1 has since been shown to induce LEM in a horse after repeated intravenous administration of the pure compound (Marasas et al., 1988). It became important to demonstrate the presence of the fumonisins and especially FB_1 in feeds associated with field cases of LEM. Voss et al. (1989) showed by thin-layer chromatography (TLC) and by liquid secondary ion mass spectrometry that both FB_1 and FB_2 were present in corn screenings that caused field cases of LEM but did not report the concentrations of the two toxins in the sample.

The liquid chromatographic technique used by Gelderblom et al. (1988) to determine concentrations of the fumonisins in cultures of *F. moniliforme* was not sensitive enough for the analysis of field samples (detection limit $10\ \mu g\ g^{-1}$). The development of a high-performance liquid chromatography (HPLC) technique by Shephard et al. (1990), which employs precolumn derivatization with *o*-phthaldialdehyde (OPA), reverse-phase separation, and fluorescence detection, has now made it possible to obtain accurate data on the levels of FB_1 and FB_2 in cereals and in feed samples. The method proved to be highly reproducible, with recoveries of the toxins from the purification steps being 99.5% and 85.9% for FB_1 and FB_2, respectively. Detection limits were $50\ ng\ g^{-1}$ for FB_1 and $100\ ng\ g^{-1}$ for FB_2.

This analytical procedure was used in the present investigation to accurately determine the levels of FB_1 and FB_2 in a number of feed samples associated with the development of histologically confirmed cases of LEM (Wilson et al., 1990). The production of FB_1 and FB_2 by 10 cultures of *F. moniliforme* isolated from these samples is also reported. These 10 cultures were selected from 100 isolates on the basis of being the most toxic to ducklings when corn cultures were incorporated in their diet.

EXPERIMENTAL PROCEDURES

Feed Samples. During a 4-year period from 1983 to 1986 samples were obtained from feeds that were fed to horses 1–3 weeks prior to development of LEM. The samples originated from various localities in the southeastern United States. In all cases LEM was confirmed by clinical signs, gross pathology, and, in some cases, histopathology (Wilson et al., 1990). Fourteen of these samples were analyzed for FB_1 and FB_2. Ten samples were commercially prepared feeds, while the other four (U6205, U6213, U6214, and U6292, Table II) were corn. Pure cultures of *F. moniliforme* were isolated from all feed samples.

Fungal Cultures. One hundred strains of *F. moniliforme* isolated in pure culture from feed associated with LEM (Wilson et al., 1990) were cultured on corn and tested for toxicity on ducklings as previously described (Marasas et al., 1979). The ten most toxic corn cultures were selected on the basis of the mortality rate, the time to death, and the feed intake causing death (Table I) and analyzed for the presence of moniliformin, FB_1, and FB_2.

Determination of FB_1 and FB_2. Fungal cultures and feed samples were analyzed for FB_1 and FB_2 by the method of Shephard et al. (1990). Samples were blended with methanol/water (3:1) and filtered, and an aliquot of the filtrate was applied to a Bond-Elut strong anion exchange (SAX) cartridge equilibrated with methanol/water (3:1). The cartridge was washed succes-

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Table I. Fumonisin Concentration and Toxicity to Ducklings of *F. moniliforme* Cultures Isolated from Feed Samples Associated with Outbreaks of Equine Leukoencephalomalacia in the United States

		toxicity to ducklings[c]			fumonisin concn, μg g^{-1}				
MRC[a] no.	FRC[b] no.	mortality, no. dead/no. tested	mean days to death	total feed intake, g	FB_1	FB_2	total (FB_1 + FB_2)	% FB_1[e]	moniliformin
4056	M2530	4/4	4.2	18	1200	150	1350	89	ND[d]
4057	M2533	4/4	4.7	18	1120	170	1290	87	ND
4058	M2534	4/4	4.7	24	1150	150	1300	88	ND
4059	M2543	4/4	5.2	18	470	20	490	96	ND
4060	M2643	4/4	4.5	22	3710	950	4660	80	ND
4061	M2648	4/4	4.7	34	3800	890	4690	81	ND
4062	M2657	4/4	4.7	36	3290	540	3830	86	ND
4063	M2570	4/4	4.2	12	520	60	580	90	ND
4064	M2610	4/4	5.0	25	160	20	180	89	ND
4065	M2462	4/4	4.7	8	270	40	310	87	ND

[a] MRC, South African Medical Research Council. [b] FRC, Fusarium Research Center. [c] Data from Wilson et al. (1990). [d] ND, not detected (<10 mg g^{-1}). [e] % $FB_1 = 100 \times [FB_1/(FB_1 + FB_2)]$.

Table II. Fumonisin Concentration of Feed Samples Associated with Confirmed Cases of Equine Leukoencephalomalacia in the United States

		fumonisin concn, μg g^{-1}			
sample	source	FB_1	FB_2	total	% FB_1[a]
U-6177	North Carolina	4.6	1.1	5.7	81
U-6178	North Carolina	4.4	0.7	5.1	86
U-6179	North Carolina	3.7	0.6	4.3	86
U-6205	North Carolina	8.0	4.1	12.1	66
U-6213	South Carolina	6.0	2.4	8.4	71
U-6214	South Carolina	27.0	12.6	39.6	68
U-6256	North Carolina	5.8	1.6	7.4	78
U-6258	North Carolina	4.5	1.0	5.5	82
U-6262	North Carolina	1.3	0.1	1.4	93
U-6263	North Carolina	9.6	3.1	12.7	76
U-6264	North Carolina	16.8	6.5	23.3	72
U-6292	North Carolina	7.1	6.3	13.4	53
U-6301	North Carolina	6.2	1.3	7.5	83
U-6302	North Carolina	3.2	0.5	3.7	86
mean		7.7	3.1	10.8	71

[a] % $FB_1 = 100 \times [FB_1/(FB_1 + FB_2)]$.

sively with methanol/water (3:1, 8 mL) and methanol (3 mL), whereafter the toxins were eluted with 0.5% acetic acid in methanol (14 mL). In the analysis of fungal cultures 5 g of culture material was extracted with 50 mL of methanol/water (3:1), and 1 mL of the extract was applied to the SAX column while the purified residue was dissolved in 1 mL of methanol before an aliquot was removed for derivatization. In the case of the feed samples 25 g was extracted with 50 mL of methanol/water (3:1), and a 5-mL aliquot of the filtered extract was applied to a SAX cartridge. The aliquot was evaporated to dryness and the residue dissolved in 0.1 M sodium borate (200 μL). Aliquots (50 μL) were derivatized by the addition of OPA solution prepared by dissolving OPA (40 mg) in 1 mL of methanol and adding 5 mL of 0.1 M sodium borate and 50 μL of 2-mercaptoethanol. Aliquots (10 μL) of the OPA derivatives were analyzed by HPLC between 1 and 2 min after derivatization. The HPLC system consisted of a M-45 pump and a U6K injector (Waters Associates, Milford, MA) connected to a 650S fluorometric detector (Perkin-Elmer, Norwalk, CT). Separations were done on a Phenomenex (Rancho Palos Verdes, CA) column (250 × 4.6 mm) packed with Ultracarb 7 μm ODS 30 reverse-phase material by using methanol/0.1 M sodium dihydrogen phosphate (80:20), adjusted to pH 3.3 with orthophosphoric acid, as mobile phase at a flow rate of 1 mL min^{-1}. Fluorescence of the eluate was monitored at excitation and emission wavelengths of 335 and 440 nm, respectively, and quantification was by peak area measurement using a data module (Waters 745) and comparison with authentic fumonisin standards.

Determination of Moniliformin. Fungal cultures were analyzed for moniliformin by the method of Thiel et al. (1982). Samples (3 g) were extracted with water (40 mL) by shaking for 1 h. The contents were then centrifuged and the supernatant extracts filtered through 0.45-μm Millipore HA filters. Aliquots of the purified extracts were analyzed by HPLC using paired ion chromatography on a reverse-phase column and UV detection at 229 nm. Quantification was done by peak area measurement and comparison with an authentic moniliformin standard.

Mycotoxin Standards. Moniliformin was isolated from cultures of *Fusarium subglutinans* (Wollenw. & Reinking) Nelson, Toussoun and Marasas as described by Steyn et al. (1978). FB_1 and FB_2 were isolated from cultures of *F. moniliforme* strain MRC 826 by the procedures described by Gelderblom et al. (1988). The identity of the toxins was confirmed by NMR spectroscopy and the purity assessed by TLC and HPLC. Only a single compound was detected in each preparation by the chromatographic procedures.

RESULTS AND DISCUSSION

F. moniliforme has long been known to be the causative fungus in the development of LEM in horses (Kellerman et al., 1988). The recent isolation of the fumonisins (Gelderblom et al., 1988) made it possible to prove that FB_1 is a causative agent in the development of this disease (Marasas et al., 1988). It remained to be proved that the fumonisins are present in feeds associated with LEM and to establish the levels at which they are present in incriminated feeds. Voss et al. (1989) reported the presence of both FB_1 and FB_2 in corn screenings that caused field cases of LEM, but gave no indication of the levels at which they were present. Earlier methods to quantify the levels of fumonisins in fungal cultures (Gelderblom et al., 1988) lacked the sensitivity to quantify naturally occurring fumonisins in cereals and feeds. The HPLC procedure developed by Shephard et al. (1990) made it possible to obtain accurate and reproducible data on the levels of fumonisins in cultures and feed samples.

All ten cultures of *F. moniliforme* isolated from feeds associated with confirmed field cases of LEM and selected

on the basis of the highest toxicity to ducklings produced both FB_1 and FB_2, but no moniliformin (Table I). The FB_1 concentration varied from 160 to 3800 $\mu g\ g^{-1}$ and the FB_2 concentration from 20 to 950 $\mu g\ g^{-1}$. There was no relationship between the toxicity of the cultures and their fumonisin content as the most toxic culture (MRC 4065, Table I), on the basis of the lowest total feed intake causing four of four deaths, had the second lowest fumonisin content. Furthermore, neither the mean time to death nor the amount of feed consumed was lower in the ducklings treated with the three cultures containing the highest fumonisin concentrations (MRC 4060, 4061, and 4062, Table I).

It is, however, significant that all cultures had the ability to produce both FB_1 and FB_2. FB_1 proved to be the major fumonisin, and its percentage of the total concentration varied between relatively narrow limits (80–96%, Table I). This is in agreement with the data on cultures of *F. moniliforme* strain MRC 826 reported by Gelderblom et al. (1988). As toxicity to ducklings does not seem to be a measure of fumonisin content, it is possible that higher producers of fumonisins could have been selected had the selection process been based on other criteria. It also appears that the toxicity of the culture material was at least partly due to a toxin other than fumonisins B_1 and B_2 or moniliformin, as no moniliformin was detected in any of the cultures (Table I).

The analytical data on the feed samples associated with confirmed cases of LEM (Table II) are in agreement with the report by Marasas et al. (1988) that FB_1 is a causative factor in the development of LEM. All 14 feeds, 10 of which were commercially prepared mixed feeds, contained both FB_1 and FB_2 with the total fumonisin concentration varying between 1.4 and 39.6 $\mu g\ g^{-1}$ (Table II). Commercially prepared horse rations have previously been linked to cases of equine leukoencephalomalacia (Wilson et al., 1985). In 7 of the 14 feeds the percentage of FB_1 fell within the range produced by cultures of *F. moniliforme* (80–96%, Table I). In the other seven feed samples relatively higher proportions of FB_2 were present, while in one sample (U-6292, Table II) FB_2 formed almost 50% of the total fumonisins.

No data exist at present on the potential of FB_2 to cause LEM, as the only animal experiments on pure compounds involved intravenous administration of pure FB_1 to a horse (Marasas et al., 1988). It is therefore impossible to predict to what extent the FB_2 in the feeds contributed to the inducement of LEM.

Minimum levels of fumonisins in feeds required to induce LEM in horses need to be established. The levels found in the present study on confirmed cases of LEM will have to be compared to future results, in similar investigations, before any firm conclusions can be made as the number of samples in the present investigation was relatively small.

The mean concentrations of FB_1 and FB_2 in the incriminated feed samples (7.7 and 3.1 $\mu g\ g^{-1}$, respectively) found in this investigation agree well with the concentrations found in a case of LEM in South Africa where the feed contained 8.85 $\mu g\ g^{-1}$ FB_1 and 3.00 $\mu g\ g^{-1}$ FB_2 (Shephard et al., 1990).

These results support the observation by Marasas et al. (1988) that the fumonisins are causative factors in the development of LEM in horses and give an indication of the levels of FB_1 and FB_2 in feeds that could precipitate the disease.

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