J Vet Diagn Invest 2:213-216 (1990)

Fumonisin B_1 levels associated with an epizootic of equine leukoencephalomalacia

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Abstract. During the fall of 1989, an episode of equine leukoencephalomalacia involved 18 of 66 purebred Arabian horses at a breeding/training stable in Arizona. Of the 18 horses affected, the condition was fatal in 14. These horses, as well as 48 unaffected horses, had been fed a diet containing a substantial amount of white corn screenings. Gross pathologic findings included liquefactive necrosis in parts of the cerebral white matter and hemorrhagic foci of various sizes in the brain stem. Histopathologic findings included rarefied white matter with pyknotic nuclei and eosinophilic cytoplasm. Thin-layer chromatography, high-performance liquid chromatography, and gas chromatography/mass spectroscopy were utilized to identify and quantitate fumonisin B_1 in 3 samples of corn from the farm. Concentrations of fumonisin B_1 range from 37 to 122 ppm. Fumonisin B_1 was also detected. Using information on diet, animal weights, and feeding practices, estimates of total fumonisin B_1 dosage were determined. This is the first definitive report on equine leukoencephalomalacia and associated fumonisin B_1 concentrations.

Equine leukoencephalomalacia (ELEM) is distributed worldwide[3,5-7] and is caused by the mycotoxin fumonisin B_1 (FB_1), a metabolite of *Fusarium moniliforme.* [6] The disease often results from feeding *F. moniliforme*-infected corn and has been associated with the consumption of commercially prepared diets.[12] Studies also indicate that metabolites from various strains of *F. moniliforme* may be hepatocarcinogenic to rats.[4,10] A recent review of the history, seasonality, clinical syndromes, epidemiology, and differential diagnosis of ELEM has been published.[8] Analytical methods for FB_1 in feeds are now evolving[9] since the isolation and chemical identification of FB_1. [1,4] Previously, only fungal isolation and pathologic findings were used to diagnose ELEM.

During the fall of 1989, an epizootic of ELEM occurred at a breeding/training stable of 66 purebred Arabian horses in Arizona. We report here the results of the clinical, pathologic, and analytical investigation of this episode. It also represents the first report of the feeding duration and ELEM-associated FB_1 levels.

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Received for publication February 12, 1990.

Materials and methods

Case history. Sixty-six horses were at the stable during the outbreak, which was characterized clinically by acute deaths with or without the various neurological symptoms (ataxia, head pressing, circling, and blindness) of 1-2 days duration. Two different batches of locally obtained white corn screenings comprised a significant portion of the ration of most of the animals. Additionally, the animals received 0.2 kg/day of a protein supplement and free choice of alfalfa or grass hay. Gross examination of the screenings revealed no obvious mold, and both batches contained cob parts, damaged kernels, and undamaged kernels. Prior to September 30, the horses did not receive any corn screenings and were fed only sweet feed. Corn screenings from Batch 1 were fed from September 30 to October 19, and screenings from Batch 2 were fed from October 19 to October 25. The corn screenings were initially mixed with the sweet feed at a ratio of 1:1 and were fed for the first 4 days (September 30-October 3) and thereafter the screenings were fed at the rate shown in Table 1. The screenings were removed from the diet at the onset of the illness (October 25). Five yearling animals did not consume any screenings, and 5 animals received only Batch 1 (approximately 19 days). None of these animals became ill. Fifty-six horses of mixed age and sex received corn from both Batch 1 (approximately 19 days) and Batch 2 (approximately 7 days); 38 were not affected, and 18 became ill over a 7-day period. Of the 18 that became ill, 14 died and 4 partially recovered but were mildly affected with impaired vision and deviated lips and noses. Necropsies were performed on 10 animals: 2 on the farm and 8 at the Arizona Veterinary Diagnostic Laboratory.

Specimen collection. Brain and other tissues were collected and fixed in 10% buffered formalin, embedded in paraffin, sectioned at 5 μm thickness, and stained with hematoxylin and eosin. The following feed samples were collected

Table 1. Patient data, screenings portion of the diet, and FB_1 dose rate for 14 horses that died during an outbreak of equine leukoencephalomalacia (ELEM).

Age	Sex	Estimated weight (kg)	Necropsy	Screenings/ day (kg)	Estimated FB_1 (mg/ kg/day)*
7 mo	M	148	None	4	1.9
2 yr	F	295	None	4	1.0
4 mo	F	136	ELEM†	4	2.1
8 mo	M	159	None	4	1.8
5 mo	M	172	ELEM	3	1.3
7 mo	M	159	None	4	1.8
8 mo	M	145	ELEM	4	2.0
5 mo	M	172	ELEM	4	1.8
8 mo	F	182	ELEM	4	1.6
2 yr	M	386	ELEM	6.1	1.1
2 yr	F	409	ELEM	4	0.7
6 yr	M	520	ELEM	4	0.6
8 mo	M	183	ELEM	4	1.6
1 yr	M	Unknown	ELEM	3.4	ND‡

* Obtained using average result (72 ppm) from 3 determinations.
† Confirmed by gross and/or histopathologic examination.
‡ ND = not done.

for chemical analyses: 1) protein supplement-2 samples, 1 each from an open container and a closed bag, 2) sweet feed-2 samples, 1 each from 2 different bags of sweet feed consisting of flaked corn, crimped barley, pellets (oats, wheat middlings, barley, corn), molasses, and rice hulls, 3) alfalfa pellets–6 samples, 1 from an open bag and 5 each from closed bags, 4) alfalfa hay–4 core samples from 4 unopened bales, 5) grass hay–4 core samples from 4 unopened bales, 6) white corn screenings– 1 sample from Batch 1 (fed from September 30 to October 18), and 7) white corn screenings-2 samples from Batch 2 (fed from October 18 to October 25).

Analytical methodology. Analytical methods for determining FB_1 in feeds were developed and are similar to those recently reported.[9] Feed samples were ground to a uniform consistency in a mill.[a] A 10-g sample was weighed into a 250-ml beaker and covered with 50 ml of acetonitrile (CH_3CN)/water (H_2O) (50/50, v/v). The beaker was covered with aluminum foil and shaken for 30-60 min. A 10-25-ml portion of the solvent was filtered through medium filter paper, and the remaining solvent was treated with common household bleach to destroy excess FB_1 (Ronald Vesonder, personal communication). Two milliliters of filtered extract was added to 5 ml of H_2O and loaded onto a C18 clean-up column.[b] The column was washed with 2 ml of H_2O and 2 ml of CH_3CN/H_2O (20/80, v/v). Fumonisin B_1 was eluted with 2 ml of CH_3CN/H_2O (50/50, v/v).

One to 5 microliters of sample was spotted on a high-performance thin-layer chromatographic (TLC) plate[c] (10 x 10 cm, normal phase) along with 100 ng of an authentic FB_1[d] standard. The plate was dried with a heat gun to remove the water from the spotting zone and then developed in 60/30/10 (chloroform/methanol [MeOH]/acetic acid) to within 1-2 cm of the top. After air drying, the plate was sprayed with p-anisaldehyde (0.5% in MeOH/sulfuric acid/acetic acid, 90/5/l, v/v/v) and heated at 100 C for 5 min. Fumonisin B_1 was observed as a red-purple spot at Rf 0.25.

In a 1–ml glass vial, 100 µl of the sample was added to 100 µl of borate buffer (0.1 M, pH 8-9) followed by 100 µl of fluorescamine[e] (0.4 mg/ml in CH_3CN). The mixture was allowed to react at room temperature for 1 min, then 0.5 ml of CH_3CN/0.01 M boric acid (40/60, v/v) was added. Twenty microliters of the mixture was then injected onto a high-performance liquid chromatograph (HPLC)[f] equipped with a fluorescence spectrometer[g] (excitation 390 nm, emission 475 nm) and a 10-cm 5-µm C18 analytical column.[h] The mobile phase was pumped at 1 ml/min and consisted of a step gradient as follows: 1) 0.1 min-39.5% CH_3CN/59.5% H_2O/1.0% acetic acid, 2) 6.0 min-49.5% CH_3CN/49.5% H_2O/1.0% acetic acid, and 3) 4.0 min- 59.5% CH3CN/39.5% H_2O/1.0% acetic acid (all solvent ratios were v/v/v). Quantitation was based on fluorometer response (peak height or peak area) compared to an authentic FB_1 standard that was reacted with fluorescamine.

One milliliter of 1.0 N potassium hydroxide was added to an aliquot of the crude sample extract (usually 1 g equivalent) in a screw-cap vial. The sample was warmed (70 C) for 1 hr. The solution was then made acidic with 0.5 N hydrochloric acid and loaded onto a 2.54-cm column of XAD-2[i] in a disposable 4.0-ml syringe barrel. The column was washed with 8 ml of H_2O, and the hydrolyzed FB_1 was eluted with a 8 ml of methanol. The methanol was evaporated under nitrogen, and 1.0 ml of MeOH was added to redissolve the sample. An aliquot of this fraction (usually 0.05 ml) was added to a screw-cap vial and evaporated to dryness with nitrogen, then 100 µl of trifluoroacetic acid (TFA) was added. One microliter was injected onto the gas chromatograph/mass spectrometer (GC/MS)[j] equipped with a 15-m fused silica capillary column.[k] The column was held at 120 C for 2 min, then programmed at 20 C/min to 200 C, and then at 10 C/min to 270 C. Identification and quantitation of the TFA derivative was based on GC/MS response compared to an authentic FB_1 standard that had undergone the identical hydrolysis, clean-up, and derivatization.

Results

Fumonisin B_1 concentrations. The screenings were analyzed by TLC, HPLC, and GC/MS, and the other feed samples were analyzed by HPLC and TLC. Results from all techniques were in agreement for all samples tested. High-performance liquid chromatography showed FB_1 retention time matching peaks with identical peak ratios to that of the standard, and GC/MS gave mass spectra for the samples identical to that of the standard. Thin-layer chromatography yielded identically colored spots for standards and samples with matching Rfs. Results of analyses on a single subsample of Batch 1 and 2 subsamples of Batch 2 were 56, 37, and 120 ppm FB_1, respectively. Other portions of the diet, 20% protein supplement, alfalfa pellets, and sweet feed contained little if any FB_1 (<5 ppm).

Fumonisin B_2 (FB_2) was detected by HPLC and GC/MS in the same extract as the respective fluorescamine

and TFA derivatives. Based on HPLC response compared with an authentic FB_1[d] standard, FB_1 levels were estimated to be 2, 11, and 23 ppm, respectively, for the samples with FB_1 results of 37, 58, and 122 ppm. Fumonisin B_2 elutes earlier on GC/MS and later on HPLC than FB_1.

To determine how the fumonisins were distributed in the screenings, a subsample of Batch 2 was separated by hand into 3 main components: 1) undamaged kernels, 2) damaged kernels, and 3) cob parts. These components were analyzed by HPLC and TLC. The damaged kernels and cob parts had respective FB_1 levels of 148 and 144 ppm and FB_2 levels of 41 and 31 ppm. The undamaged kernels contained <5 ppm each of FB_1 and FB_2.

Pathologicfindings. Gross examination of all horses necropsied showed focal (10 x 20 mm) to diffuse unilateral areas (30 mm x 6 cm) of liquefactive necrosis in various areas of the cerebral white matter (Fig. 1). Portions of the cerebrum in some animals disintegrated when removed from the cranial vault. Hemorrhagic foci of various sizes were often present in the brain stem. Histopathologic lesions included rarefied white matter with pyknotic nuclei and eosinophilic cytoplasm. Some sections had no identifiable tissue structures, whereas other sections often contained variable-sized hemorrhagic foci located in a distinct perivascular pattern. Although both gross and microscopic lesions were common in the cerebrum, severe hemorrhagic lesions were also observed in the brain stem in some cases.

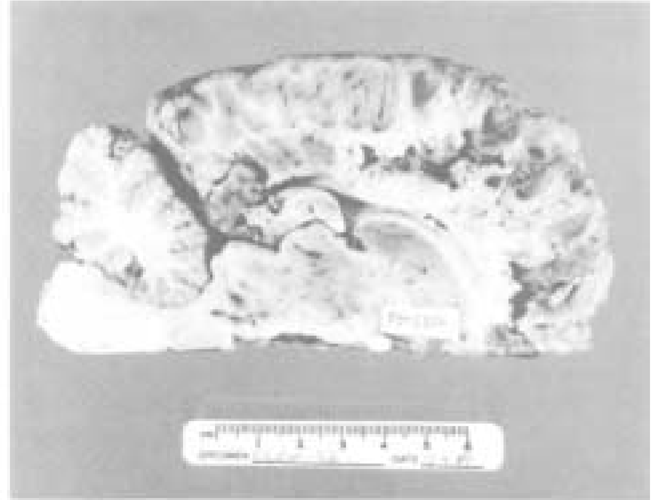

Figure 1. Sagittal section of brain of horse with leukoencephalomalacia.

Discussion

At the time of the outbreak, only GC/MS analytical methodology for detection of FB_1 was known to be under development.” Using chemical data on FB_1 from the original isolation and characterization work[1,4] and taking advantage of the FB_1 amino function, the TLC and HPLC methods were developed to complement GC/MS. Subsequently, TLC and HPLC methods similar to those described here have been reported,[9] with some notable exceptions: 1) CH_3CN/H_2O is used instead of $MeOH/H_2O$ (FB_1 is unstable in MeOH when stored for extended periods of time), 2) CH_3CN is used as the solvent for fluorescamine as opposed to acetone (FB_1 is practically insoluble in acetone, making the derivatization difficult), 3) C18 clean-up columns are used for clean-up thereby providing a cleaner extract than the reported silica based procedure, and 4) high-performance TLC plates are used to significantly improve resolution and sensitivity (FB_1 streaks on conventional silica TLC plates, reducing sensitivity and resolution).

Comparison of interlaboratory data on TLC, HPLC, and GC/MS replicate analyses of feed samples containing FB_1 (from sources other than here) has shown consistent results. A rigorous precision and accuracy study is currently in progress. Analytical work with FB_2 is limited, but further study is planned.

With the data obtained in this study, we cannot identify Batch 1 or 2 of the corn screenings as the primary source of FB_1. Fumonisin B, levels suggest both were involved. Batch 2 was consumed closest to the onset of the outbreak, but FB_1 concentrations were not uniform, as exhibited by the range obtained from 2 subsamples (37 and 122 ppm). Only a single sample was obtained from Batch 1. Even though there was obvious variation in FB_1 levels, dose rates in Table 1 were calculated from the amount of screenings fed and are based on 72 ppm (the mean of the 3 determinations) and the estimated animal weights. Precision measurements on control samples clearly show this variation was not entirely due to method variance, which was typically < 10%. This concentration (72 ppm) is consistent with or higher than levels detected in feeds from 16 other ELEM cases that this laboratory was involved with during the fall of 1989 (P. F. Ross, unpublished data) and with a previous report.[6] Comparison of the estimated dose levels to published values is difficult as only intravenous dosing has been reported (0.125 mg/kg body weight for 9 days).[7] The feeding duration of 26 days is consistent with reports of 28 and 30 days in ELEM studies using *F. moniliforme* culture material where FB_1 levels were not known.[2,11] This is the first report detailing FB_1 levels. The meaning of the FB_2 levels is not clear because toxicity information is not known.

The large number of horses that received screenings but were not affected is difficult to explain. It is not clear whether this was due to varying FB_1 content or some other factor, although the clinical attack rate, 18

of 61 (23%), and the case fatality rate, 14 of 18 (68%), are similar to those of the previous reports[3] (Wilson TM, Nelson PE, Marasas WFO: 1989, Abstr Annu Meet AAVLD #29).

This laboratory studied 16 cases of ELEM during the fall of 1989; a higher number than in previous years. Whether this represents an increase in the incidence of the disease due to some undetermined factar(s) associated with corn cultivation, weather, and/or the characteristics of *F. moniliforme* or whether it was due to increased awareness must be determined. The finding that FB_1 and FB_2 occurred in the damaged kernels and cob parts but not in the undamaged kernels suggests that contaminated corn may be made safe by a thorough screening process. The case reported here represents the first field case study of ELEM with documented concentrations of FB_1 with known feeding duration. Using the described reliable analytical procedures and available FB_1 standard, research can now progress to answer questions about ELEM.

Acknowledgements

We acknowledge the assistance and continued support of Dr. Delmar R. Cassidy and Dr. Robert M. Nervig and the technical contributions of Dr. Paul E. Nelson, James N. Shively, Darcy D. Schackelford, Rod Fichtner, and Michele McLeod.

Sources and manufacturers

a. Stein Mill Model M-l, Fred Stein Labs, Inc., Atchison, KS.
b. Sep-pak (TM), Waters, Division of Millipore, Milford, MA.
c. Whatman LHP-K HPTLC Plates, P. J. Cobert Assoc., Inc., St. Louis, MO.
d. Division of Food Science and Technology, Pretoria 001, South Africa.
e. Aldrich Chemical Co., Milwaukee, WI.
f. Perkin-Elmer, Model 250 LC Pump, Norwalk, CT.
g. Perkin-Elmer, Model LS-3 Fluorescence Spectrometer, Norwalk, CT.
h. Brownlee RP-18 Spheri-5 Analytical Cartridge, P. J. Cobert Assoc., Inc., St. Louis, MO.
i. Amberlite (TM) XAD-2, Mallinckrodt, Inc., St. Louis, MO.
j. Hewlett-Packard Model 5970 (MSD), Hewlett-Packard, Avondale, PA.
k. 0.25 mm DB-1, J and W Scientific, Folsom, CA.

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