Hazards Presented by Mycotoxins and Toxigenic-Mold Spores in Feeds

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Received January 24, 1974; accepted April 16, 1974

Hazards Presented by Mycotoxins and Toxigenic-Mold Spores in Feeds. CARDEILHAC, P. T. AND NAIR, K. P. C. (1974). Toxicol. Appl. Pharmacol. 30, 299-308. The prevalence of cytotoxic CHCl₃-soluble mycotoxins and toxigenic-mold spores was evaluated in 997 feeds entering or prepared in a large feed mill during one calendar year. Mycotoxins were obtained as either a feed lipid fraction by CHCl₃ extraction of the feed, or a mold lipid fraction by CHCl₃ extraction of Czapek-Dox broth after a 7-day culture of mold spores present in the feed. Mycotoxins were detected by a bioassay which determined cytotoxicity of the sample to chick tracheal ring organ cultures. Aflatoxins were determined by thin-layer chromatography. Feed lipids comprised 1.1-3.0% of the wet weight of the samples and were assayed by suspending the lipid from 0.10 g of feed in 1.0 ml of culture medium, producing a mean lipid concentration of 1917 μg/ml. A 100% cytotoxic response was given in 16% of the lipid samples from nine basic feed types. Based on results from calculated dose-response curves, cytotoxic mold lipids which did not contain aflatoxin were more than 30 times as toxic as feed lipids, and mold lipids which contained aflatoxin were more than 300 times as toxic as feed lipids. Feed samples were suspected of contamination with mycotoxins when both feed and mold lipids were cytotoxic. Mycotoxin contamination was suspected in 5.7% of 749 samples with over half of the suspect samples containing aflatoxin. All samples contaminated with aflatoxin contained less than 200 parts per billion.

The mycotoxicoses include a diverse group of diseases which affect several organ systems in various ways; therefore, clinical signs and lesions produced by them may vary with the particular toxin involved (Pier, 1973). The difficulty in diagnosing mycotoxicoses by clinical signs, lesions, history, or other clinicopathological methods makes it difficult to determine the incidence and seriousness of these diseases.

The purpose of the present study was to better evaluate the potential hazards presented by mycotoxins; consequently, it was decided to estimate the prevalence of mycotoxins and spores of molds capable of producing mycotoxins in feeds. With the exception of aflatoxin, standard analytical methods for the mycotoxins are complicated and pure reference compounds for all mycotoxins are not available. A general method of multimycotoxin analysis based on solvent extraction and chemical identification was not feasible. Therefore, a simple inexpensive general test was designed which was based on chloroform extraction of the sample and detection of toxin in the extract by

1 Florida Agricultural Experiment Station Journal Series No. 5262.
bioassay for cytotoxicity. The presence of aflatoxins in the extract was indicated by thin-layer chromatography (TLC) and confirmed with bioassay. Viable mold spores in the sample were cultured in a mold-selective broth (Czapek-Dox) which was extracted with chloroform after culture and the extract tested for the presence of mycotoxins.

**METHODS**

**Sample Collection**

Samples were collected from virtually all feed entering a large feed mill during one calendar year (November 1970 to November 1971). More than 20 samples for each of nine types of single-component feeds (the nine basic feeds) were collected. Samples of the nine basic feeds were consistent in composition, and did not present special difficulties in analysis. A total of 632 samples of the nine basic feeds were collected (identities of the nine basic feeds and numbers tested are given in table 3). Less than 20 samples of other feeds and feed ingredients such as soybean meal, molasses, milo, dicalcium phosphate, bagasse pellets, and urea were collected. Dried citrus pulp and other material, such as waste products from the manufacture of candy, were screened but not considered as basic feed.

Samples taken from carloads were obtained by collecting approximately 1 kg of sample at some point during the transfer of the feed from the car into the mill. Samples of mixed feed were obtained by collecting approximately 500 g of sample from 100 feed sacks containing the finished feed. All samples used (997) were kept in plastic bags at room temperature (23°C), and processing began within 2 weeks of collection. Individual samples were mixed, finely ground, and two aliquots of 2.5 and 0.02 g were collected. The 2.5-g aliquot of finely ground feed was extracted with chloroform; while the 0.02-g aliquot was the source of mold spores for culture.

**Feed Lipids**

Thirty milliliters of chloroform-water (1:1) mixture were added to the 2.5-g aliquot in a 250-ml flask, and extraction was accomplished by vigorous shaking. This chloroform extract is referred to as feed lipids.

**Mold Lipids**

The 0.02-g aliquot was added to 15 ml Czapek-Dox broth and incubated at room temperature for one week in a cotton-plugged 250-ml flask with a ground joint. After incubation, 15 ml of chloroform was added to the broth, the cotton plug was replaced with a closely ground glass stopper, and extraction accomplished as before. The chloroform extract of the broth is referred to as mold lipids.

**Sample Preparation**

The chloroform extract and washings were separated from the aqueous phase and filtered by means of phase separation paper. Chloroform was removed from the extract with a steam bath, the dry feed and mold lipids were reconstituted to 0.5 ml in chloroform, and portions were removed for TLC, bioassay, and a determination of the dry weight.

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2 DIFCO Laboratories, Detroit, MI.
3 Whatman No. 1PS.
**TLC**

Aliquots (0.025 ml) of feed and mold lipids were applied, usually to adjacent scribed channels on plates precoated with silica gel, and the plates were developed with chloroform–acetone (90:10 v/v) in unlined, unequilibrated tanks. Aflatoxin mixtures present in the lipid sample migrated slightly less than an aflatoxin B1 standard (Rf approximately 0.5). Quantities of aflatoxin in the samples were estimated by visual comparison of fluorescent intensities with the standard. For grain-based feeds, a fluorescent intensity equal to approximately 20 ppb of aflatoxin B1 in the feed was found to be the minimum amount detectable by this procedure. An estimated accuracy of comparing fluorescent intensities has been reported to be approximately ± 28% (Beckwith and Stoloff, 1968).

**Bioassay**

Tracheal ring explants were exposed to feed or mold lipid, suspended in tissue culture medium, and any toxic effect on ciliary movement detected by a comparison with controls. The procedure and methods for the estimation of a dose–response curve have been previously described (Nair et al., 1970; Cardeilhac et al., 1972).

After chloroform was removed from an aliquot (10%) of the sample of feed lipid and either a 10, 5 or 2% aliquot of the mold lipid sample, the residue was suspended in 0.05 ml of ethanol, which had been saturated with 0.2 M sodium phosphate buffer (pH 7.2), and mixed with 2.5 ml of tissue culture medium. An aliquot of the mixture was added to a culture dish containing 2–4 tracheal rings 0.1 mm thick. The rings and controls in medium which contained no lipids were incubated 48 hr in a 5% CO2 incubator at 37°C. Ciliary movement in the rings was evaluated by viewing with an inverted microscope. A 100% cytotoxic response was the complete loss of ciliary movement, while a 50% response would reduce ciliary movement to approximately one-half of the controls. Dose–response curves were approximated by a least-squares fitting of percent response versus the logarithm of lipid concentration in the same manner that curves had previously been estimated by fitting percent response versus pure toxin concentration (Nair et al., 1970). It is possible to obtain a negative slope by this method since the lipid concentration does not reflect the toxin concentration.

**Cytotoxicity**

The maximum mean concentration of feed lipid which was not cytotoxic was estimated for each of the nine basic feeds and for mixed feed by determining the mean concentration of feed lipid obtained from 10 or more samples which produced no cytotoxicity at concentration ranges where at least 20% of all samples of the feed type being tested produce some cytotoxicity (Table 1). A feed lipid was considered cytotoxic if it produced a 100% cytotoxic response at concentrations in the culture medium approximately equal to or less than the determined maximum mean concentration which was not cytotoxic. Mold lipids were considered cytotoxic if they produced either a 100% cytotoxic response or produced detectable cytotoxicity at concentrations less than 79 μg/ml. The concentration of 79 μg/ml was an ED50 from a dose–response curve calculated for 44 samples of mold lipids which produced detectable cytotoxicity.
# TABLE 1

**CYTOTOXICITY OF FEED LIPIDS TO CULTURES OF TRACHEAL RINGS FROM DAY-OLD CHICKS**

<table>
<thead>
<tr>
<th>Feed</th>
<th>No. tested</th>
<th>Lipid concentration* (ppm)</th>
<th>Cytotoxicity level</th>
<th>None detectable No. (%)</th>
<th>Intermediate No. (%)</th>
<th>100% loss of ciliary movement No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cottonseed hull</td>
<td>134</td>
<td>1358 ± 126</td>
<td></td>
<td>104 (77.6)</td>
<td>21 (15.7)</td>
<td>9 (6.7)</td>
</tr>
<tr>
<td>Soy millfeed</td>
<td>67</td>
<td>1568 ± 170</td>
<td></td>
<td>27 (40.3)</td>
<td>21 (31.3)</td>
<td>19 (28.4)</td>
</tr>
<tr>
<td>Hominy</td>
<td>130</td>
<td>2573 ± 174</td>
<td></td>
<td>103 (79.2)</td>
<td>21 (16.2)</td>
<td>6 (4.6)</td>
</tr>
<tr>
<td>Brewers grain</td>
<td>62</td>
<td>2255 ± 207</td>
<td></td>
<td>28 (45.2)</td>
<td>11 (17.7)</td>
<td>23 (37.1)</td>
</tr>
<tr>
<td>Peanut meal</td>
<td>50</td>
<td>1135 ± 157</td>
<td></td>
<td>29 (58.0)</td>
<td>12 (24.0)</td>
<td>9 (18.0)</td>
</tr>
<tr>
<td>Cottonseed meal</td>
<td>19</td>
<td>2000 ± 408</td>
<td></td>
<td>14 (73.7)</td>
<td>3 (15.8)</td>
<td>2 (10.5)</td>
</tr>
<tr>
<td>Corn</td>
<td>25</td>
<td>1982 ± 242</td>
<td></td>
<td>19 (76.0)</td>
<td>1 (4.0)</td>
<td>5 (20.0)</td>
</tr>
<tr>
<td>Oats</td>
<td>21</td>
<td>3048 ± 392</td>
<td></td>
<td>10 (47.6)</td>
<td>4 (19.1)</td>
<td>7 (33.3)</td>
</tr>
<tr>
<td>Wheat bran</td>
<td>18</td>
<td>2158 ± 577</td>
<td></td>
<td>10 (55.6)</td>
<td>4 (22.2)</td>
<td>4 (22.2)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>526</strong></td>
<td><strong>1917 ± 75</strong></td>
<td></td>
<td><strong>344 (65.4)</strong></td>
<td><strong>98 (18.6)</strong></td>
<td><strong>84 (16.0)</strong></td>
</tr>
</tbody>
</table>

* Cytotoxicity was evaluated by comparing the effect of media containing feed lipid with a control.

* The concentrations are expressed as mean ± SE.
among 199 samples of mold lipids obtained from mixed feeds. Cytotoxic mold lipids indicated the presence of toxigenic-mold spores in a feed sample.

Detection Limits for Mycotoxins

Based on results obtained from bioassay of chromatographically pure mycotoxins added to tissue culture medium (Cardeilhac et al., 1972), any concentration, in ppm of mycotoxins, in the feed greater than the following would be detected: sterigmatocystin, 1.2; aflatoxin B, 2.2; gliotoxin, 8.5; aflatoxin G, 8.9; sporidesmin, 14; sporidesmin B, 16; ochratoxin A, 18; patulin, 64; aflatoxin B₂, 137. These detection limits assume a 100% extraction of toxin from the feed but disregard any potentiation of toxicity by other agents present in the extract. In control samples of grain-based mixed feed, to which aflatoxin B₁ had been added, substances in the feed lipid were found to potentiate the toxicity and thus reduce the minimum concentration detectable in some cases to less than 0.1 of the listed concentration. For example, concentrations of aflatoxin B₁ as low as 20 ppb were detected in some of these control samples.

Mycotoxins

A feed sample was suspected of containing mycotoxins when both feed and mold lipids were cytotoxic. The cytotoxicity of feed lipids was believed to result from causes other than mycotoxins if toxigenic-mold spores were not found.

Toxigenic-mold Spores

Samples were considered to contain toxigenic-mold spores when the mold lipids were cytotoxic.

Aflatoxin

A sample was determined to contain aflatoxin when the feed lipids were cytotoxic and contained material with fluorescent and chromatographic properties similar to the aflatoxin standard. Quantities of aflatoxin were estimated by TLC fluorescent intensities.

Spores of Molds Capable of Producing Aflatoxin

A sample was determined to contain spores of molds capable of producing aflatoxin when the mold lipids from the sample were cytotoxic and had material with fluorescent and chromatographic properties similar to the aflatoxin standard.

RESULTS

Quantities of Lipid Extraction

The mean dry weight of feed lipids obtained from 18 or more samples per feed type for each of the nine basic feed types ranged from 1.1 to 3.0% of the sample. Feed lipid comprised 0.2% of mixed feed samples tested. The mean dry weight of mold lipids, obtained from culture in 15 ml of Czapek-Dox broth of 0.02-g aliquots from 18 or more feed samples for each of the nine basic feed types ranged from 11 to 24 mg.

Toxicity of Feed Lipid

A 10% aliquot of feed lipids obtained from 2.5-g samples of the nine basic feeds added to 2.5 ml of culture medium produced no detectable cytotoxicity in 65.4% of 526
samples for which complete data were available (Table 1). For individual feed types, the percentage of samples which produced no detectable toxicity ranged from 40.3 to 79.2%. No detectable cytotoxicity was found in 62 % of feed lipids from 117 samples of mixed feeds. A 100 % cytotoxic response was found in 84 (16.0 %) feed lipids from the nine basic feeds and in 11 (9.4 %) of the 117 mixed feeds samples. An intermediate cytotoxic response (< 100%) was obtained in 98 (18.6%) of 526 samples of feed lipids from the nine basic feeds and in 26 (22.2%) of 117 samples of feed lipids from mixed feeds. The mean concentrations of feed lipids from the nine basic feeds which produced a 100 % cytotoxic response were identical to the mean concentrations which produced no detectable cytotoxicity (2029 µg/ml).

**Aflatoxin**

One-hundred-five (16.5%) of 634 samples of feed lipid from the nine basic feeds, for which chromatographic and cytotoxic data were complete, had fluorescent material with chromatographic properties similar to aflatoxin; while 26 (4.1%) of the fluorescent samples were cytotoxic and thus determined to contain aflatoxin. One sample of peanut meal, with a concentration of approximately 200 ppb, was the only sample found to have an aflatoxin concentration greater than 100 ppb. For 825 samples composed of the nine basic feeds and mixed feeds, 42 (5.1%) were determined to contain aflatoxin and 57 (6.9%) contained spores of molds capable of producing aflatoxin. Three of the 825 samples (0.4%) were determined to contain both aflatoxin and spores of molds capable of producing aflatoxin.

**Toxicity of Mold Lipids**

The cytotoxicity of mold lipids was evaluated from data obtained from 516 samples of the nine feeds for which complete data were available (Table 2) and from usable data from all 997 samples of feeds and mixed feed. As experience from the biological assay of mold lipids was gained, the size of the aliquot of mold lipids tested was reduced from 10 to 5% and finally to 2% of the chloroform extract. With a reduction in the size of the aliquot, the percentage of cytotoxic responses reduced from 62.4% for the 10% aliquot to 4.3% for the 2% aliquot. As the size of the aliquot was reduced, the portion of cytotoxic mold lipids which contain aflatoxin increased, from 16% for the 10% aliquot to 33% for the 2% aliquot. The mean concentration produced by all aliquots of mold lipid from 202 cytotoxic samples found among 516 samples of the nine basic feeds was 630 µg/ml. The concentration of mold lipid produced by adding a 2% aliquot to the culture medium ranged from 0.8 to 118 µg/ml for 349 samples tested. A mean mold lipid concentration of 19 µg/ml was found for 15 samples with detectable cytotoxicity found among this group. Seven of the 15 samples produced lipid concentrations of less than 4 µg/ml in the culture medium and also produced a detectable cytotoxicity to the tracheal explants. Three of the seven contained aflatoxin.

A dose–response curve for cytotoxicity was approximated with data from 48 samples of mold lipids which contained aflatoxin. A positive slope was obtained and the calculated ED50 concentration was 6.8 µg/ml. Another dose–response curve was approximated in the same manner using 44 2% aliquots of mold lipids from mixed feeds which were cytotoxic but did not contain aflatoxin, and the calculated ED50 was 79 µg/ml.
TABLE 2

CYTOTOXICITY OF MOLD LIPIDS TO CULTURES OF TRACHEAL RINGS FROM DAY-OLD CHICKS*

<table>
<thead>
<tr>
<th>Feed</th>
<th>No. tested</th>
<th>Lipid concentration(^b) (ppm)</th>
<th>Cytotoxicity level</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>None detectable No. (%)</td>
<td>Intermediate No. (%)</td>
</tr>
<tr>
<td>Cottonseed hulls</td>
<td>134</td>
<td>504 ± 87</td>
<td>65 (48.5)</td>
</tr>
<tr>
<td>Soy millfeed</td>
<td>65</td>
<td>748 ± 163</td>
<td>34 (52.3)</td>
</tr>
<tr>
<td>Hominy</td>
<td>127</td>
<td>616 ± 102</td>
<td>55 (43.3)</td>
</tr>
<tr>
<td>Brewers grain</td>
<td>60</td>
<td>649 ± 147</td>
<td>27 (45.0)</td>
</tr>
<tr>
<td>Peanut meal</td>
<td>46</td>
<td>814 ± 270</td>
<td>20 (43.5)</td>
</tr>
<tr>
<td>Cottonseed meal</td>
<td>19</td>
<td>625 ± 221</td>
<td>7 (36.8)</td>
</tr>
<tr>
<td>Corn</td>
<td>25</td>
<td>421 ± 119</td>
<td>12 (48.0)</td>
</tr>
<tr>
<td>Oats</td>
<td>22</td>
<td>689 ± 223</td>
<td>10 (45.5)</td>
</tr>
<tr>
<td>Wheat bran</td>
<td>18</td>
<td>608 ± 180</td>
<td>10 (55.6)</td>
</tr>
<tr>
<td></td>
<td>516</td>
<td>618 ± 51</td>
<td>240 (46.5)</td>
</tr>
</tbody>
</table>

* See Table 1.
\(^b\) Concentrations are expressed as mean ± SE.
TABLE 3

THE PREVALENCE OF AFLATOXIN IN FEED OR MOLD LIPIDS FROM FEED SAMPLES SUSPECTED OF CONTAINING MYCOTOXINS

<table>
<thead>
<tr>
<th>Aflatoxin</th>
<th>Feed</th>
<th>Brewer grain (69)</th>
<th>Corn (29)</th>
<th>Cotton seed hulls (165)</th>
<th>Cotton seed meal (32)</th>
<th>Hominy (158)</th>
<th>Soy mill-feed (74)</th>
<th>Peanut meal (58)</th>
<th>Oats (25)</th>
<th>Wheat bran (21)</th>
<th>Mixed feed (117)</th>
<th>All samples (749)</th>
<th>% of all samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>4</td>
<td>5</td>
<td>5</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>22</td>
<td>2.9%</td>
</tr>
<tr>
<td>-</td>
<td>5</td>
<td>1</td>
<td>5</td>
<td>2</td>
<td>0</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>21</td>
<td>2.8%</td>
</tr>
<tr>
<td>Total</td>
<td>6</td>
<td>2</td>
<td>7</td>
<td>4</td>
<td>4</td>
<td>8</td>
<td>7</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>43</td>
<td>5.7%</td>
</tr>
</tbody>
</table>

* Both feed and mold lipids from the feed sample were cytotoxic.

b Numbers in parentheses indicate number of samples tested.
Mycotoxins in Feeds

The presence of mycotoxins was evaluated for 632 samples of the nine basic feeds and 117 samples of mixed feeds (Table 3). Forty-three of these 749 samples (5.7%) were found to have both feed lipids and mold lipids which were cytotoxic upon initial screening and thus were suspected of possible contamination with mycotoxins. Twenty-two of the 43 suspicious samples contained aflatoxin in either the feed or mold lipids, and thus 21 were suspected of containing other mycotoxins.

DISCUSSION

The present study offers evidence of the importance and potential importance of chloroform-soluble mycotoxins in feeds at the time they enter the mill. Although some difficulties were encountered with certain feeds, the procedures seemed adequate for the nine basic feeds studied and for mixed feeds composed from these basic feeds. It is acknowledged that all possible mycotoxins may not be extracted by the procedures used and some of those extracted may not be detected by the bioassay used. However, many toxins should be at least partially extracted and detected, at levels required to produce acute lesions in animals, based on the properties of mycotoxins presently characterized (Lillehoj et al., 1970). It should be further acknowledged that toxigenic-mold spores found in the present study may never have vegetated under even poor conditions of feed storage and nontoxigenic-mold spores could have overgrown potentially toxigenic-mold spores under the special conditions of this study. There also may be a question concerning detection limits. Feed lipid obtained from 0.1 g of feed is the limit that can be added to culture medium used in the present bioassay without producing a high incidence of false positives. This limitation sharply reduces the sensitivity of the assay when the detection limit is based on concentrations of pure mycotoxin assayed in the absence of feed lipids; however, the presence of feed lipid was found to increase sensitivity more than 10-fold in some cases. Unfortunately, no reliable factor to account for increased sensitivity could be developed because of the large variance produced by differences in lipid composition and concentrations. Dose–response curves developed by fitting percent response versus logarithm of lipid concentration are subject to error because the lipid concentration does not reflect toxin concentration. However, this method seemed the best and certainly the most familiar way to objectively analyze the data. Although these and other possibilities for error exist in a study of this nature, a number of conclusions are suggested concerning the hazard presented by mycotoxins in feeds.

It is clear that most molds from spores present in feeds can produce chloroform-soluble products that are several times more toxic than chloroform-soluble substances present in feed before mold growth. Molds from spores present in 4.3% of the feed samples produce lipids which are approximately 30 times more toxic than feed lipids. Mold lipids containing aflatoxin are approximately 300 times more toxic than feed lipids. Thus, molds not presently considered highly toxigenic often produce toxic products in addition to removing readily accessible required nutritional factors from the feed.

* Some samples (116) with incomplete data which were not used in Table 2 could be used in this analysis.
In this study, over half of the feeds suspected of possible contamination with mycotoxin contained aflatoxin or spores of molds capable of producing aflatoxin. The other half of the suspect feed samples had lipids which were considered cytotoxic and contained spores of molds capable of producing lipids which are cytotoxic at concentrations roughly equal to mold lipids containing aflatoxin. The importance of these molds as causes of mycotoxicosis if the feed were stored under poor conditions is unknown. For feeds containing aflatoxin the concentrations were less than 100 ppb in all but one sample and approximately 200 ppb in this sample. If the day-old duckling is considered the most susceptible animal, consumption of a feed containing 200 ppb aflatoxin B₁ for less than 1 week will not produce severe acute lesions in animals. Thus, with dilution and a probable time of exposure of less than 1 week for individual feeds it seems likely that any feed, at least those found to contain aflatoxin, in the present study would require a period of storage under conditions favourable for mold growth to present any acute hazard to livestock. The importance of aflatoxin as the principle hazard among the mycotoxins tends to be confirmed by this study, and this finding emphasized the importance of reliable methods for aflatoxin detection.

There was no statistically significant difference in the incidence of mold spores capable of producing aflatoxin among all feed samples and those determined to contain aflatoxin. This result was unexpected and cannot be fully explained, although many of the feeds, such as oil seed meals, are processed and spores of the molds which originally produced toxin may be removed before arrival at the mill. Also, the concentrations of aflatoxin present in feeds used in this study are well below those required to produce acute toxicity and thus below those for which the assay procedure was designed to reliably detect. Only three of 825 samples (0.4 %) were determined to contain aflatoxin and spores of molds capable of producing aflatoxin.

REFERENCES


