Management of Aflatoxin Contaminated Maize in Tamaulipas, Mexico

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A rural warehousing facility in the state of Tamaulipas in Mexico stored aflatoxin contaminated maize from 1989 to 1995; its control required national efforts. The aflatoxin contaminated maize was analyzed chemically by immunoaffinity columns, fluorometry, and thin layer chromatography in grain, weeds, and soil of the warehouse "Las Yescas". Morphological and tributyrine biochemistry tests were done to identify species of *Aspergillus* in grain, weeds, and soil, and mainly *A. flavus* was found. The average levels of total aflatoxin found were 66 μg/kg from 42 samples of sieved maize, 295 μg/kg from 49 samples of not sieved maize, 25 μg/kg from seven weed samples, and 112 μg/kg from nine soil samples. Sieving reduced the aflatoxin by 75%. Soil always presented AFB_2, and sometimes AFB_1, due to the high contamination.

Keywords: *Aflatoxins; fluorometry; immunoaffinity; contamination; Aspergillus flavus; TLC; carcinogenic; environmental toxicology*

INTRODUCTION

Aflatoxins (AF) are secondary metabolites of the fungi *Aspergillus flavus*, *A. parasiticus*, and *A. nomius* that chemically correspond to bis(dihydrofuran) coumerins, and their chemical properties are well-known (Asao et al., 1963; Butler, 1974; Chang et al., 1963).

Maize is the staple food of the Mexicans, and Tamaulipas is an important producer and suffered a strong AF contamination in this cereal for several years, 440 000 tons just in 1991, both at field and at storage places. The Mexican Government has spent around 2 million U.S. dollars yearly to develop the Aflatoxin in Maize Detection Program of the State of Tamaulipas, where all the maize crop of this State was analyzed, around 20 000 AF chemical analysis every year (Juan et al., 1995). Tamaulipas has a rural warehouse called Las Yescas (Figure 1) that was chosen by the Mexican Government to store all the AF contaminated maize from 1989 to 1995. This warehouse is in the district of Valle Hermoso and belongs to Rural Warehouses of the National Commission of Popular Subsistance, or Conasupo, now under the Secretary of Agriculture.

Huge mounds of contaminated maize (>60 000 tons) were simultaneously stored here; the use of this contaminated maize for food is illegal (in 1993, around 5000 tons were left outside in the open). The remaining 55 000 tons had been sold or mixed with the soil. Two methods for decontamination were used: with ammonium, 20 000 tons, by a U.S. company from Arizona, and another 20 000 tons with aluminosilicates by a Mexican private company. These two practices were abandoned because the cost of the decontamination was higher than economical recovery.

It was decided to bury part of the maize using tractors because the Secretary of Urban Development and Ecology (SEDUE) had forbidden burning of such grain because the spores would have disseminated everywhere increasing the problem. Later, the remaining maize was sieved with proper sieving machines that discarded all of the powder, and some of it was re-used as animal feed without proper dilution. There were many complaints by the farmers around Las Yescas because many chickens died, and the owners thought that AF had contaminated the mantle water deposits and caused the problem when the water rose into their wells. This protest was not scientifically based.

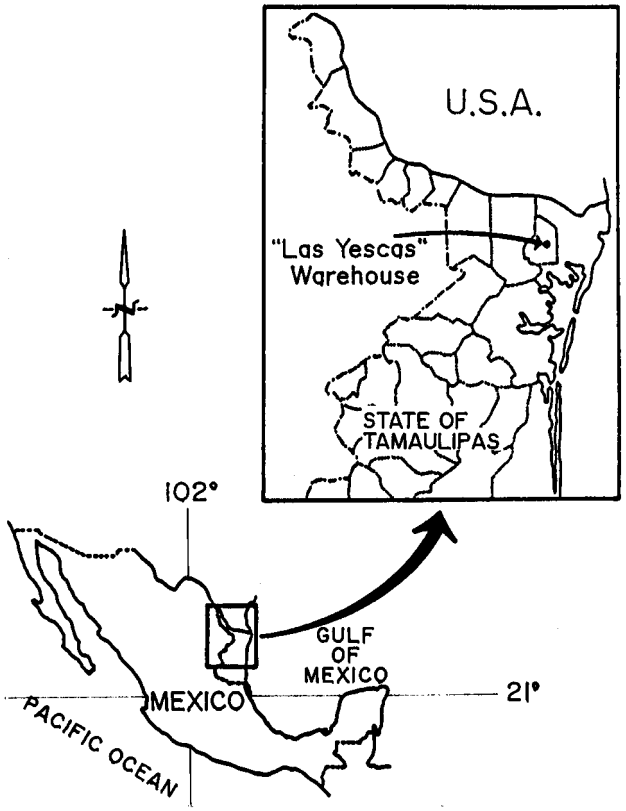

Figure 1. Geographical situation of the rural warehousing facility called Las Yescas in the state of Tamaulipas, Mexico.

The aims of the research are the following:

(a) To examine the viability of *Aspergillus* in the Las Yescas warehouse.

(b) To identify the *Aspergillus* species with biochemical and morphological studies.

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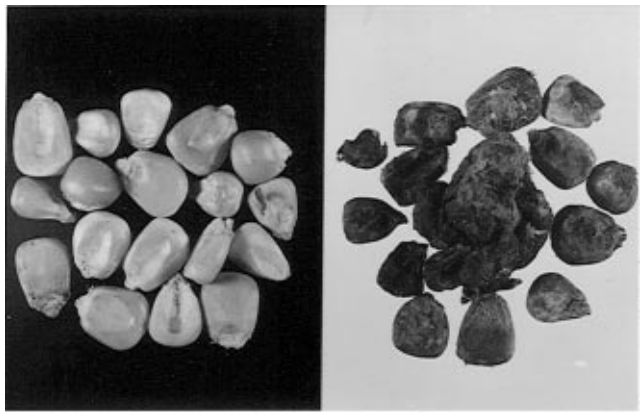

Figure 2. AF contaminated maize (A) sieved and yellow and (B) nonsieved and black from the warehouse Las Yescas, Tamaulipas, Mexico.

(c) To quantify the AF present in 107 samples: 91 of maize, six of weeds, and nine of soil from Las Yescas to certify the contamination level.

MATERIALS AND METHODS

A. flavus and *A. parasiticus* contaminate corn in the field and grow as saprophytes in crop debris. Sclerotia of *A. flavus* is the source of primary inoculum in damaged and healthy maize grains. The toxigenic strains share many attributes. An analysis based on both morphology and chemistry was useful.

Culture Media. Malt salt agar (MSA) culture media adjusted to a pH 4.6 was used to isolate Aspergillus fungi (Tuite, 1969). Other taxonomic indicators to differentiate *A. flavus* from *A. parasiticus* were colony growth on agar plates, following Raper and Fennell (1965) criteria based on the temperature and composition of the culture media, age, color of aerial parts, growth diameter, and colony edges.

To sterilize the medium, a steam pressure boiler (American Sterilizer Co.) at 110 °C and 20 pounds of pressure during 20 min was used. The petri dishes were sterilized in an electrical dispatch oven at 180 °C for 1 h. Petri dishes with MSA medium were incubated at 28 °C for 2 days to ensure aseptic conditions.

Fungi Isolation from Grains. Maize grains sieved and not sieved from Las Yescas were placed in filter papers with 5 mL of water inside petri dishes in sterile conditions, to locate the fungus in the grain. In a second assay, the grains were disinfected previously with sodium hypochlorite (at 2%) and placed in MSA petri dishes at 25 °C for 4 days. The assays were one seed (not sieved) of "black" maize from the outside of the mound or five seeds of sieved maize per petri dish (Figure 2). Ten replications were done with and without disinfection. The isolated colonies were stained with acid fuchsin and identified under microscope.

Fungi from Soil and Weeds. Samples of Las Yescas soil were analyzed both from the surface and at a depth of 30 cm, to determine if the fungus was still alive and AF present; the AF contaminated maize was buried in the soil with tractors in 1990, considering that this treatment could break down such toxins based on the literature (Angle, 1987). To isolate the fungi strains, soil dilutions (1:1000 and 1:10 000) in sterile distilled water were done. One milliliter of each dilution was placed in MSA with 10 replications.

The genus *Paspalum* sp., family Poaceae, was the only weed present and was analyzed to check for the presence of *Aspergillus*; seven samples of 50 g each were chemically analyzed for AF. Five dry weed segments of 4 mm were placed in MSA medium and incubated at 28 °C for 72 h to obtain the fungal colonies.

Fungus Identification Tests. *Biochemistry Tests to Identify Lipolytic Strains.* To identify lipolytic strains (*A. parasiticus*) from the *A. flavus* that are not, a biochemical test with tributyrine was done (Magan et al., 1993; Mosmuller et al., 1962; Yang and Chen, 1994). Translucent halos around the lipolytic fungal colonies are formed because they metabolize the lipid called tributyrine, and the rest of the medium remains opaque. No other lipid must be present in the medium to avoid false positives.

The tributyrine agar medium contains meat peptone (2.5 g/L), casein peptone (2.5 g/L), yeast extract (3.0 g/L), agar-agar (12 g/L), tributyrine from Merck (10 mL/L), and water. After being poured into petri dishes, fungal spores were incubated for 72 h at 28 °C. Ten replications of *Aspergillus* spp. colonies were isolated from sieved maize, soil, and weeds.

Morphological Study. To identify the isolated fungi, photographs from the different asexual stages of *Aspergillus* were taken. The width and length of 20 spores were measured to help as a taxonomical criteria, as Raper and Fennell (1965) considered.

Chemical Analysis. (a) A biocode immunoaffinity concentration procedure for extraction and quantitation of AF was done and detected by fluorometry (Carvajal et al., 1990). The method used was as follows: 50 g of maize, weed, or soil was blended for 2 min with 5 g of sodium chloride and 100 mL of methanol (80:20 v/v) dissolved in water and filtered using Whatman paper no. 1. Three milliliters of the filtrate was dissolved in 12 mL of PBS buffer and refiltered in glass wool filter paper, and 10 mL was applied to the column. Each immunoaffinity column was prepared separately, receiving 20 mL of PBS at a rate of 5 mL per minute, not permitting the column to dry. The column received 10 mL of the refiltered sample; it was washed with 20 mL of distilled water, and it was dried by passing air thru. AF were eluted slowly from the column with 1.5 mL of pure methanol, collecting the eluate into a cuvette. Molecular bromine (1 mL) was added to the cuvette as a developing solution. The fluorometer was calibrated using Biocode standards, and AF amounts (μg/kg) in each sample were read in the fluorometer.

(b) Thomas, Eppley, and Trucksess (1975) method. Thin layer chromatography (TLC) method was done to determine the kind of AF present in the samples. TLC permits the segregation and identification of aflatoxins AFB_1, AFB_2, AFG_1, AFG_2, AFM_1, and AFM_2 (Table 2).

The method was as follows: 50 g of maize was blended with 250 mL of a methanol/water solution (60:40 v/v) at high speed for 2 min. The content was filtrated thru Whatman no. 4 filter paper and was collected in a 250 mL separation flask. This last received 30 mL of a saturated solution of sodium chlorate and 50 mL of hexane, shaking and degasifying for 1 min. When the two phases were separated, the methanol−water phase was transferred to another separation flask and the upper organic remainder phase was driven away. To extract methanol, 50 mL of chloroform were added, mixed, and degasified. The lower phase (with chloroform) was transferred to a 250 mL flask, mixing with 5 g of copper carbonate to precipitate the pigments. When the copper carbonate sedimented, the mixture was filtered through 5 g of anhydrous sodium sulfate and received in a 125 mL flask. The copper

Table 1. Quantitation of Aflatoxins from the Different Samples of the Warehouse Las Yescas, Tamaulipas, Mexico, in 1993

	no. of different kinds of contaminated aflatoxins samples			
amount (μg/kg)	sieved maize[a]	nonsieved maize[b]	weeds[c]	soil[d]
1−20	2		5	
21−50	19			
51−100	15	6	2	4
101−200	5	12		5
201−300	1	11		
301−400		12		
401−500		5		
501−600		2		
610−2000		1		

[a] Total AF averages of 42 samples of sieved maize = 66 μg/kg. [b] 49 samples from not sieved maize = 295 μg/kg. [c] Seven samples of weeds = 25 μg/kg. [d] Nine samples of soil = 112 μg/kg.

Table 2. Determination of the AF Types in TLC, from Some Samples of Maize and Soil from the Rural Warehouse Las Yescas, Tamaulipas, Mexico

no.	sample	total AF (μg/kg)	AF type
25	sieved	156	B_1
26	sieved	153	B_1, B_2
36	sieved	42	B_1, B_2
	Average of Three Samples[a]		
65	not sieved	357	B_1
69	not sieved	448	B_1, B_2
83	not sieved	97	B_1, B_2
84	not sieved	468	B_1
	Average of Four Samples[b]		
92	soil	82	B_2
93	soil	181	B_1, B_2
94	soil	51	B_2
95	soil	104	B_2
96	soil	169	B_1, B_2
97	soil	174	B_1, B_2
98	soil	71	B_2
99	soil	106	B_2
100	soil	69	B_2

[a] Average of three samples of sieved maize with TLC = 117 μg/kg of total AF. [b] Average of four samples of not sieved maize with TLC = 343 μg/kg of total AF.

carbonate remaining in the flask was washed again with 25 mL of chloroform and passed thru anhydrous sodium sulfate again, collecting it in the same flask. The extract was dried to 1 mL and transferred to a clean vial, where it was dried completely with liquid nitrogen. The extract was redissolved in 1.5 mL of methanol in a vial, vortexed for 1 min, and applied to TLC Merck plates (0.2 mm silica width) in 10, 20, 30, and 50 μL sample spots, and the four AF standards were applied. The developer was a mixture of acetone (17%), ethyl acetate (33%), and toluene (50%).

RESULTS

Fungal Strains from Maize Grains, Weeds, and Soil. The best growth of an olive green fungus was obtained from the "non-disinfected" sieved maize. The black external maize was so damaged that it was not a nutritional medium for fungal growth.

With isolates taken from the yellow sieved grain grown in MSA medium, a successful growth of pure olive green colonies of *A. flavus* was obtained in 2 days. The fungus was present both inside and outside the grains.

From soil and weed samples, a colony growth was observed after 24 h of incubation. After 72 h, the colonies were clearly distinct. Isolates from *A. flavus* with their typical olive green color were obtained both from soil and weed sections. Other dark ivy green colonies of *A. parasiticus*, *Penicillium* spp., and other different kinds of fungi (*Rhizopus* spp. and *Fusarium* spp.) were also isolated.

This assay was completed with the examination of the fungi stained with acid fuchsin, where conidia, mycelia, biserial sterigmata, and sporangia typical from *A. flavus* were found in all the isolated colonies.

Biochemistry Identification Tests. *Lipolytic Strains.* The colonies sown in the medium with tributyrine gave negative results without a translucent halo surrounding the limits of the colony; after 72 h of incubation at 28 °C, the medium remained opaque.

These results showed that the colonies of the fungi isolated from grain, soil, and weeds from the rural warehouse of Las Yescas were of *A. flavus* because they did not have the lipolytic activity of *A. parasiticus*.

Morphological Study. The identification of the genus *Aspergillus* based only on size and shape of the asexual spores was not conclusive; there is a taxonomical prox imity among the species to segregate, and the size of the conidia can change with age and development, from elliptical to spherical. Elliptical smooth surface spores, presence of biseriate conidiophore arrangement, and the yellow-green color of the studied colonies taken from yellow sieved maize ensures it to be *A. flavus* in an early developmental stage. Raper and Fennell (1965) reported that *A. parasiticus* have verruculose sphaerical spores.

Chemical Analysis. The amounts of total AF from yellow sieved maize, black nonsieved maize, weeds, and soil from Las Yescas are shown in Table 1. The AF quantities are variable, from 15 to 1971 μg/kg.

Only seven samples, two from sieved maize and five from weeds, were within legal limits of tolerance by Mexican and U.S. laws that established 20 μg/kg of total AF as top limit for human food; 98% of the samples had higher contamination than 20 μg/kg. The average numbers of total AF (μg/kg) detected were 66 μg/kg from 42 samples of sieved maize, 295 μg/kg from 49 nonsieved maize samples, 25 μg/kg from seven weed samples, and 112 μg/kg from nine soil samples. This confirms that most of the AF is in the maize powder.

Results of AF Identification with the Thomas–Eppley–Trucksess (1975) TLC Method. Several colorful spots appeared, but only two fluorescent blue spots with the Rf of AFB_1 and AFB_2 were taken into account. TLC from the most contaminated samples of sieved grain and nonsieved grain showed a higher presence of AFB_1 in relation to AFB_2; opposite results were obtained at soil that had mainly AFB_2 in contrast (Table 2).

DISCUSSION

This maize was sold to a private company that makes liquor for humans in Aguascalientes, Mexico; there is no quality control of food or beverages. AF are soluble in ethanol, and they can pass to the liquor easily and are a public health risk. Although Angle (1987) reported that soils have more *A. parasiticus* colonies than *A. flavus*, we found mainly the last because of the thousands of tons of contaminated grain that had been buried in the soil, in 1990, that could have changed the normal mycoflora.

The harmful effects caused by AF have been well described (Austwick, 1983; Essigmann et al., 1977; Harrison et al., 1993; Purchase, 1967; Reye et al., 1963; Smith, 1982; Wogan, 1990), although sometimes the causal agents are AF derivates and not the AF themselves.

Even as animal feed the problem is huge (Giralt-Pont et al., 1989; Hagler and Hamilton, 1982; Pier, 1979). In 1991, they pigmented the contaminated maize in dark pink, but nobody gave information to the cattle producers about the way to mix this maize with other clean ingredients and many intoxications happened. Part of this contaminated maize was sent to the city of Merida, Yucatan, presumably to "dilute it", but it was sold to animal feed companies and from there to the rest of the country. The contaminated AF maize was also sold in 1994 and 1995 for human consumption.

When bovines are fed with AFB_1 contaminated feed, it is metabolized to AFM_1 and aflatoxin M_2 (AFM_2) (Holzapfel et al., 1966), which are highly carcinogenic and stand pasteurization, milk production decreases,

and it is a health risk. The rate of transformation of AFB_1 to AFM_1 is 1% of the AF ingested by the cow (Van Egmond, 1983; Masri, 1969). A low pH 6.5 condition and the presence of metals such as Mn^{2+}, Mg^{2+}, and Va^{2+} favor the formation of hydroxylated AF derivates as AFM_1 and AFM_2 (Stein, 1980).

Sieved maize had 75% less AF than nonsieved grain, and most of the AF was in the maize surface and in the dust. The amount of AF found in soil samples after years indicated that the aflatoxigenic fungus, well mixed with the soil of Las Yescas, was still alive and that it either produced AF at 30 cm deep inside or retained the existing ones. These findings are in agreement with longevity data of *A. flavus* in corn, reported by Hesseltine and Rogers (1982), where a survival up to 10 years was found. Loam soil and, more slowly, a silty clay loam soil allow a conjugate to be formed with a reduced rate of decomposition. Las Yescas' soil is a silty clay loam soil, and maybe the AF−clay conjugate was formed; the fungus was still alive, forming more toxin because AFB_1 was present in three samples. Angle (1987) considered that few if any adverse environmental consequences would be expected from the introduction of AF into soil, and this was the criteria followed by the Conasupo authorities. But damages of AF for crops exist and are well established (Joffe, 1969; Crisan, 1973; Reiss, 1971; Black and Altschul, 1965; Lilly, 1965; Wyllie and Morehouse, 1978). So the fact of having high amounts of AF in the soil might be dangerous for future crops.

Maybe the contaminated corn in the soil preserves better the AF than the AF alone in it. No AFG_1 or AFG_2 was found, so the presence of aflatoxigenic strains of *A. parasiticus* was not meaningful. Some *A. flavus* only produce AFB_2 (Papa, 1977), but none produce only AFG. In our case the strain produced both AFB_1 and AFB_2 because they both appeared. High levels of AFB_1 have been associated with low maize yields caused by drought stress (Wallin, 1987), the situation present at Tamaulipas.

The prevalence of AFB_2 in the soil can be caused by decomposition of AFB_1 to AFB_2, a less toxic form but still dangerous. Angle (1987) reported that, when AFB_1 was added to the soil, it rapidly decomposed to AFB_2 in 4 days, which is more stable and was degraded slowly in 77 days, and that AF degraded more quickly in fertile silt soils. In our case AFB_2 was still present after 4 years.

Goldberg and Angle (1985) found that AFB_1 and its derivates were retained within the upper 20 cm of all soil types, no AF was found in the leachate from any of the soils, and no AFB_1 contamination of the groundwater would be expected to occur unless soils were sandy or shallow. In our case we found AFB_1 at 30 cm inside the soil, and we think that is risky.

After 5 years of the presence of AF contaminated maize in the soil, these toxins and the fungus can still be found. Neither zearalenone nor trichothecenes were found.

ABBREVIATIONS USED

AF, aflatoxins; AFB_1, aflatoxin B_1; AFB_2, aflatoxin B_2, AFG_1, aflatoxin G_1; AFG_2, aflatoxin G_2; AFM_1, aflatoxin M_1; AFM_2, aflatoxin M_2; Rf, retention factor; PBS, phosphate buffer solution; sp., spp., species; MSA, malt salt agar.

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