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Article

Characterization of *Aspergillus flavus* and quantification of aflatoxins in feed and raw milk of cows in Aguascalientes, Mexico

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Abstract:

Contamination of agricultural and livestock products with aflatoxins (AF) is distributed worldwide. AFs are toxic, carcinogenic, and immunosuppressive; however, in Mexico, there is little information about *Aspergillus flavus*, the main fungus that produces them. The objective was to characterize the molecular and morphological, aflatoxigenic isolates of *A. flavus* and quantify the AFs in the feed and in the milk of Holstein cows in Aguascalientes (Mexico). A dairy production unit (2,749 cows) was selected for reasons of convenience, and monthly samples of food ingredients and total mixed ration (n= 267), raw milk (n= 288), and agricultural soil (n =40) were collected during 24 months and were cultivated (in PDA) using the pour plate technique with serial dilutions. The fungi were characterized using SEM, TLC and vapors of ammonium in coconut agar; the genes of calmodulin and a regulator of the biosynthetic pathway of AF, as well as the region of the internal spacer of the transcript, were sequenced. AFs were quantified in feed with HPLC and in milk, using ELISA. A total of 283 fungal isolates were characterized molecularly; of which 88 proved to be *Aspergillus spp*. Five of these were *A. flavus* with an aflatoxigenic capacity, and one was non-aflatoxigenic. 99.3 % of the samples of feed and 39.9 % of the milk samples exhibited detectable levels of AF (14.8 and 0,021 µg/kg). The cows ate daily 621 µg of AF and eliminated 0.09 % as AFM_1 in milk. This suggests that the occurrence of aflatoxigenic *A. flavus* in the feed of dairy cows leads to a widespread contamination of the diets and food chain with AF.

Key words: Aflatoxins, *A. flavus*, Dairy foods, Calmodulin gene, Regulatory gene of aflatoxin.

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Introduction

Mexican bovine dairy is developed especially in a biogeographic province known as the Mexican Central High Plateau[(1)]. Within this region, dairy production units (DPU) face insufficiency issues in the production, economic profitability and safety of the products[(2)]. Among the food safety problems, the presence of aflatoxins (AF) has been highlighted because they cause a strong economic impact associated with the contamination of agricultural crops, a deterioration in the health of animals, a decline in productivity, and contamination of food of animal origin[(3)]. AF have hepatotoxic, nephrotoxic and

immunosuppressive properties and are considered as the most powerful known natural carcinogens[4]. AF have been quantified in feed, milk and milk products intended for food for the Mexican human population[5-7]. This suggests that the presence of AFs in the feed of cows is a common problem in the DPUs that leads to contamination of the milk with the metabolites of AF.

When dairy cows consume agricultural products contaminated with AF, the enzymatic mechanisms bioactivate the mycotoxin through the formation of an epoxide that reacts with the cellular structures and nucleic acids, damaging their integrity[4]. The reactive epoxide can also be neutralized by conjugation with glutathione or its excretion as a metabolite that is eliminated in the milk mainly as aflatoxin M_1 (AFM_1); furthermore, AFM_1 is considered as a toxic and carcinogenic agent for humans[4].

AF are secondary metabolites of several filamentous fungi of the genus *Aspergillus*, which are distributed worldwide and contaminate a large variety of agricultural products, particularly cereals[8]. Also *A. flavus* has been identified in Mexico, both in agricultural soil and in corn grains[9,10]. Asexual reproduction of *Aspergillus* species has been described previously[11,12], and so has its phylogeny, morphology and biological cycle[8,13]. Although *Aspergillus flavus* is considered to be the species with the greatest capacity for production of aflatoxins[14], other strains of *A. flavus* without aflatoxygenic capacity, as well as other species of *Aspergillus*, produce AFs (*A. parasiticus*, *A. nomius*, *A. pseudonomiu*, *A.arachidicola*, *A. bombycis*, *A. minisclerotigenes*, *A. pseudotamarii*, and *A. Togoensis*)[15]. Aflatoxygenic capacity is expressed mainly under conditions of environmental stress[16,17], as long as its genotype includes the information involved in the metabolic production chain of AF[18,19]. A strategy for the molecular identification of the different species of the genus *Aspergillus* is the use of the gene of calmodulin (CaM), the fragments corresponding to the region of the internal transcribed spacers (ITS) and the regulatory gene of the aflatoxin (AfIR) biosynthetic pathway[8,20].

The objective of this work was to characterize the molecular and morphological, aflatoxigenic isolates of *Aspergillus flavus* obtained from dairy production units of the Central High Plateau of Mexico.

Material and methods

Study area

The present study was performed with a descriptive, longitudinal and non-experimental design. A dairy production unit was selected for convenience, using the non-probabilistic method, and was followed for 24 mo. The DPU was located in the Central High Plateau of Mexico (21°48'N, 102°03' W; 1986-2008 m asl), with a dry temperate and a semi-warm semi-dry climate, with summer rains, average temperature of 18.4 º C, an annual rainfall of 518.4 mm, and a maximum altitude of 2,300 m asl.

During the observation period, the DPU had an average of 2,749 Holstein cows housed in confinement within open-air pens enclosed with metal fences, shaded areas and freely accessible feeders. The cows were milked with automated equipment, obtaining an average daily production of 28.1 L per cow. The milk production is exported to agro-industrial plants in the region. Diets were developed as a total mixed ration (TMR) in a feed mill, where the corn silage and the concentrate were blended in mixer wagons. The TMR release is formulated to meet the nutritional requirements of dairy cows. The corn for silage is obtained directly from the agricultural areas of the UPL, while the protein-energy concentrate was purchased from the Local Association of Dairy Farmers of Aguascalientes, using as main ingredients canola, soybean, corn, rolled, sorghum, soybean meal, corn distilled dry grains, cottonseed, alfalfa and oats hay, as well as premixed vitamins and minerals.

Sample collection and handling

A total of 288 samples (1.0 kg) were obtained: total mixed ration (TMR), concentrated feed and corn silage were dried up in a forced air circulation oven (OF-22G-TECH, JEOI Lab Companion, Corea). The samples were pulverized (500-800 µm) in a continuously operating universal mill (MF series 10 Basic, IKA®-Werke, Germany) and stored in cooling system (4-5 °C) until processing (<7 d). During the (morning and evening) milking, a total of 288 samples of raw milk were obtained directly from the collector tank (500 ml) for each production batch (high, medium and low). The samples were transported in refrigeration and were preserved in freezing (-20 °C) until processing (<7 d).

Five agricultural plots were sampled with the help of a soil punch, choosing four sampling points on the surface of each plot of land and taking five 100 g sub-samples at each point, at a depth of 3-30 cm. The five sub-samples were gathered in a zip lock bag. Finally, they were screened (500-800 µ) and kept in refrigeration until processing.

Quantification of aflatoxins

Food samples were analyzed according to the official method 990.33 of the AOAC[21], using solid phase columns (SPE; SupelcleanTM LC-18 SPE tube, Sigma-Aldrich, USA). The eluate extracted from the samples derivatized with trifluoroacetic acid was analyzed by HPLC (detection limit 2 µg/kg) with a fluorescence detector (binary Pump Vary Pro Star; FP 2020 detector, Varian Associates Inc., Victoria, Australia), a C18 column, and a column guard (LC-18 LC-18; Thermo Fisher Scientific, Massachusetts, USA). The quantification data were obtained using the Galaxie software (version 1.9.302.530), and AF concentrations were calculated using standard curves of purified AFs (Sigma-Aldrich, St. Louis, MO, USA). The AFM1 was quantified in raw milk with the competitive ELISA technique using a commercial kit (Ridascreen fast® aflatoxin M1 R-1121, R-Biopharm, Germany; detection range 0.005-0.08 µg/kg). The samples were homogenized and centrifuged according to the manufacturer's instructions. The absorbance was measured at 450 nm in an ELISA microplate reader (BioTek Instruments, Inc., USA). The results were interpreted upon the basis of the calibration curve made with purified 1 AFM (Sigma-Aldrich, St. Louis, MO, USA).

Fungal Isolation and characterization

The fungi were isolated using the pour plate technique with four serial dilutions in sterile peptone water[22]; the samples were seeded in a potato, dextrose and agar (PDA), malt extract with rose Bengal and Czapeck medium, and incubated (28 °C) in the darkness during 7 days. The colonies were isolated and purified, and the microscopic morphological characteristics consistent with the description of the genus were identified[13]. Isolates consistent with *A. flavus*[8,13] were submitted to the process of fixating with glutaraldehyde (2%), gradual alcohol dehydration, and conditioning with a critical point dryer (Samdri Tousimis Research 795, Rockville, Meryland) and a metalizer (Desk II, Denton Vacuum, USA) were digitized in a scanning electron microscope (JEOL JSM-5900 LV,JEOL, USA) and conducted 10

measurements of each structure (stipe, gallbladder, spore, esclerocio and fialide), and using a JEOL software (Scanning Electron Microscope). The morphological structures of the isolates identified were compared against known strains of *A. flavus*, known as AF-36, AF-Cuatitlan (AF-C) and AF-Tamaulipas, (AF-T)[14.23]. The strains obtained in this study were recorded at the NCBI (National Center for Biotechnology Information) with the respective access codes.

The aflatoxigenic capacity of the isolates was characterized by thin-layer chromatography (TLC)[24]; silica gel plates without fluorescence indicator were utilized (Z265829, Sigma-Aldrich, USA) activated in a high temperature oven (OF-22G-TECH, JEOI Lab Companion, Corea). The plates with purified standards of AFs (6636-50MG, Sigma Aldrich, USA) were placed inside of a chromatographic chamber with mobile phase chloroform - acetone - isopropanol (85:10:5, v/v/v) for an hour and a half. Eventually, the dry plates were visualized in a transilluminator. The technique ammonium vapors in coconut agar technique was also used according to the methodology described above[25]. Monosporic isolates were inoculated in sterile coconut agar and were left to incubate in the dark (30 °C, 5-7 d). Subsequently, ammonium hydroxide (200 µl; J.T. Baker, Mexico) at 25 % was added to the cover of the Petri dishes, and the distribution and the intensity of the color was observed. The presence of color was taken as indicative of AF production.

Molecular analysis

The genomic DNA of monosporic cultures of *Aspergillus* spp. was extracted according to previously standardized methods[26]. The quality of the obtained DNA was displayed with electrophoresis (45 min to 85 volts) in agarose gel (1%) with a TAE 1X damper and was quantified by comparing against known concentrations of DNA of phage λ (Thermo Fisher Scientific, MA USA). The DNA visualization was performed using a device for photoanalysis (Bio-Rad Gel Image®- Doctm XR, CA USA) with the Quantity One software version 4.6.7.

An amplification of a fragment corresponding to the region of internal spacers of the transcript (ITS**;** ITS1-5.8S-ITS2 RNAr**)** and ITS4 (5´-TCCTCCGCTTATTGATATG -3´) was carried out in accordance with the previously described protocols[27,28]; the gene of calmodulin (CaM) was amplified with the primers CMDA7F (5'-GCCAAAATCTTCATCCGTAG-3') and CDMA8R (5'-ATTTCGTTCAGAATGCCAGG-3') and the regulatory gene of the aflatoxin biosynthetic pathway (aflR) was amplified using the primers aflR-F (5'-GGGATAGCTGTACGAGTTGTGCCAG aflR-3') and aflR-R (5'-TGGKGCCGACTCGAGGAAYGGGT-3') of Eurofins Genomics, Louisville KY, USA. The polymerase enzyme Go-Taq (Promega, Madison, WI USA) and a model 9700 thermal

cycler (Applied Biosystems) were utilized in the amplification. The PCR products obtained (ITS, calmodulin and aflR) were separated by electrophoresis in (1%) agarose gel and they were observed using as intercalating agents SYBR® Gold and Orange DNA Loading Dye (Thermo Fisher Scientific, MA USA); the resulting bands were observed in an image analysis device (BIO-RAD IMAGE® GEL Molecular- DOCTM XR CA, USA) with the Quantity One software (version 4.6.7). Molecular weight marker ladders (Axygen Biosciences, CA, USA) were included. PCR products were purified with the reagent ExoSAP-IT® PCR Product Cleanup (Afflymetrix, Thermo Fisher Scientific Inc. Santa Clara, California, USA). The purified PCR products were sequenced in forward and reverse chains with the dideoxy method[29]. The samples were injected into a sequencer (ABI 3730XL Genetic Analyzers), and the resulting sequences were recorded in an electropherogram. The electropherograms were visualized with the Chromas Lite software and were compared with the records of the NCBI using BLAST (Basic Local Alignment Search Tool).

Statistical analysis

The quantitative data of the total mixed ration, milk production, AF concentration in feed and milk, and of the measurements of the structure of each isolate were subjected to a one-way variance analysis (ANOVA), considering as separate factors the time of year and the level of milk production (high, medium or low) in which the cows were classified in batches. The differences between the means and the 95 % confidence intervals were determined using the honestly significant difference (HSD) Tukey's test; $P<0.05$ was considered significant for all statistical analyses.

Results

Frequency of *Aspergillus* spp

It was identified a total of 283 fungal isolates; 56.8 % came from samples of feed for dairy cows, and the rest, from the agricultural land (Table 1). A total of 88 isolates (31.1 %) exhibited morphological features corresponding to those described for the genus *Aspergillus*. Figure 1 shows the conidiophores and conidia, and the characteristic metulae. Isolates were also found with a morphology compatible with the genera *Penicillium*, *Fusarium*, *Rhizopus*,

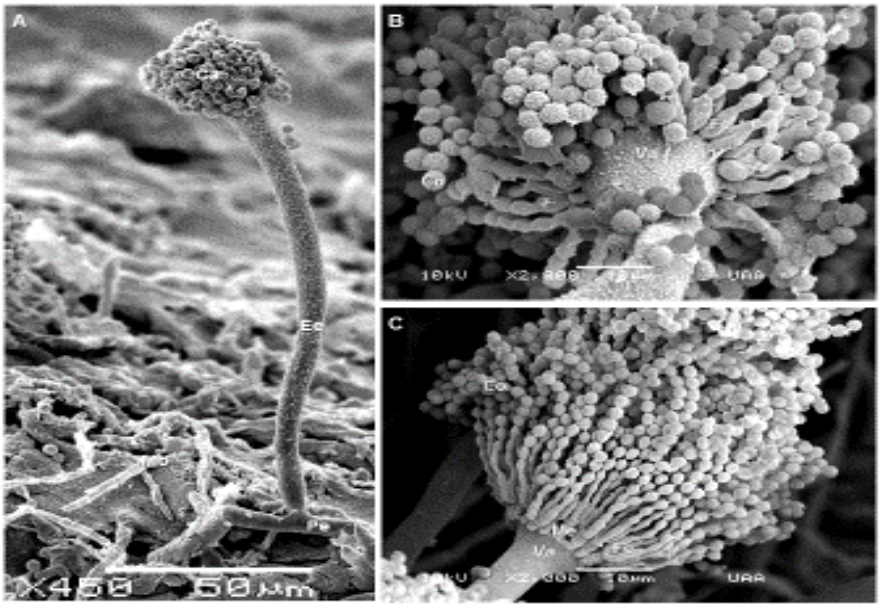
Mucor, *Cladosporium*, *Richoderma*, *Alternaria*, *Curvularia* and *Bipolaris*. The proportions of each gender are shown in Table 1.

Table 1: Fungal genera identified in monthly samples * of a dairy production unit in the Central High Plateau of Mexico

Gender	Soil		Corn silage		Feed concentrate		Total mixed ration		Total	
	No.	%	No.	%	No.	%	No.	%	No.	%
Samples	40	--	96	--	96	--	96	--	328	--
Aspergillus	30	24.6	16	42.1	19	32.8	23	35.4	88	31.1
Penicillium	23	18.9	3	7.9	4	6.9	9	13.8	39	13.8
Fusarium	20	16.4	3	7.9	11	19.0	2	3.1	36	12.7
Rhizopus	15	12.3	4	10.5	7	12.1	8	12.3	34	12.0
Mucor	15	12.3	6	15.8	4	6.9	9	13.8	34	12.0
Cladosporium	5	4.1	4	10.5	10	17.2	10	15.4	29	10.2
Trichoderma	10	8.2	0	0.0	0	0.0	0	0.0	10	3.5
Alternaria	4	3.3	2	5.3	1	1.7	2	3.1	9	3.2
Curvularia	0	0.0	0	0.0	2	3.4	1	1.5	3	1.1
Bipolaris	0	0.0	0	0.0	0	0.0	1	1.5	1	0.4
Total	122	100	38	100	58	100	65	100	283	100

*Sample of food ingredients: 24 months, in quadruplicate. Soil samples: 5 plots, in quadruplicate, 2 seasons.

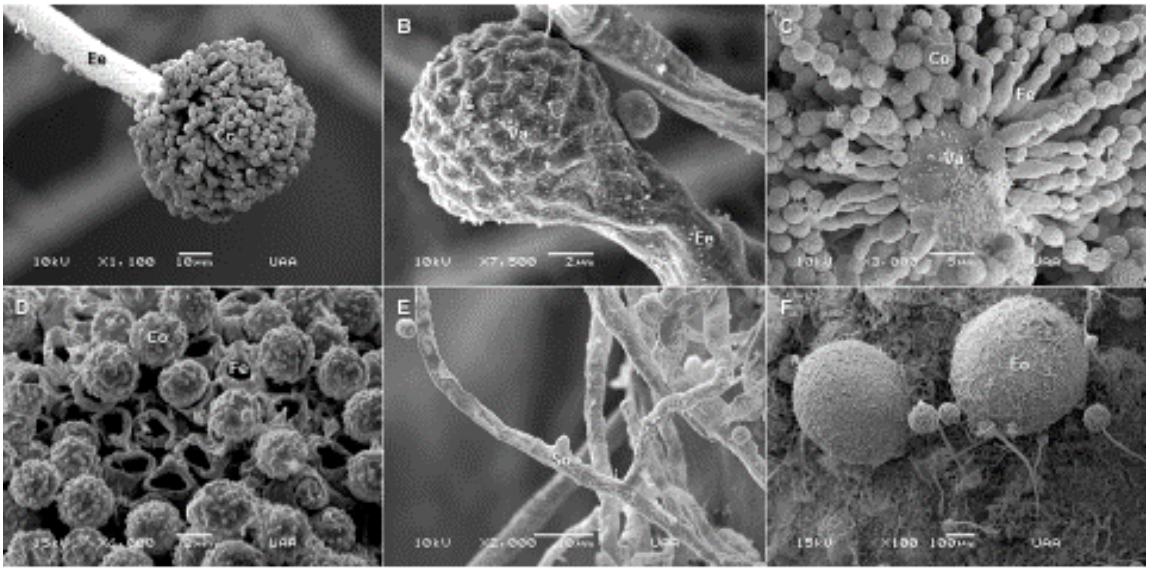
Figure 1: *Aspergillus* morphological structures



Panels: A) Conidiophore: *Cc* = conidial head, *Ee* = Stipe, *Pe* = foot cell, *Mo* = mycelium. B) conidial head uniseriate portions: *Co* = Conidium or spore; *Fe* = Phyalid, *Va* = Vesicle C) Biseriate conidial head: *Co* = Conidium, *Fe* = Phyalid , *Va* = Vesicle, *i* = Metula.

Only six isolates exhibited a morphology consistent with *A. flavus* and came from the feed concentrate (AC1, AC2 and AC3) and corn silage (EM1, EM2, EM3); when cultivated in PDA, they exhibited an olive green hue with a whitish periphery. Most of the isolates (5/6) exhibited the presence of sclerotia of dark brown color; only one did not manifest these structures. At a microscopic level, conidiophores of *A. flavus* exhibited irradiate and uniseriate conidial heads, the stipe with rough walls, the spherical vesicle, globe-shaped conidia with irregularities in their surface, and a whitish, septate mycelium (Figure 2).

Figure 2: Morphological structures of *Aspergillus flavus*



Panels: A) *Cc* = conidial head, *Ee* = stipe. B) *Va* = vesicle. C) Conidiophore. *Fe* = phyalids, *Co* = conidium. **D)** *Cc* = conidium, *Fe* = phyalid. **E)** *Mo* = mycelium, *So* = septum. **F)** *Eo* = Sclerotium.

The six isolates of *A. flavus* from feed exhibited significant differences in the morphometry of their structures when compared against the control strains (AF-C and AF-T; Table 2). These strains presented conidial heads, with rough radiated stipe and spherical vesicles, while AF-C only exhibited (uniseriate) phyalids and (biseriate) AF-T phyalids metulae; the conidia were globe-shaped with irregularities on the surface. Four strains isolated were classified as L (long sclerotium, >400 µm), one as S (short sclerotium, <400 µm), and one as without sclerotium.

Table 2: Comparison of the dimensions of the morphological structures of *Aspergillus flavus* isolates obtained from corn silage (CS) and whole feed (WF) for dairy cows, as well as of the Cuautitlan (C) and Tamaulipas (T) strains

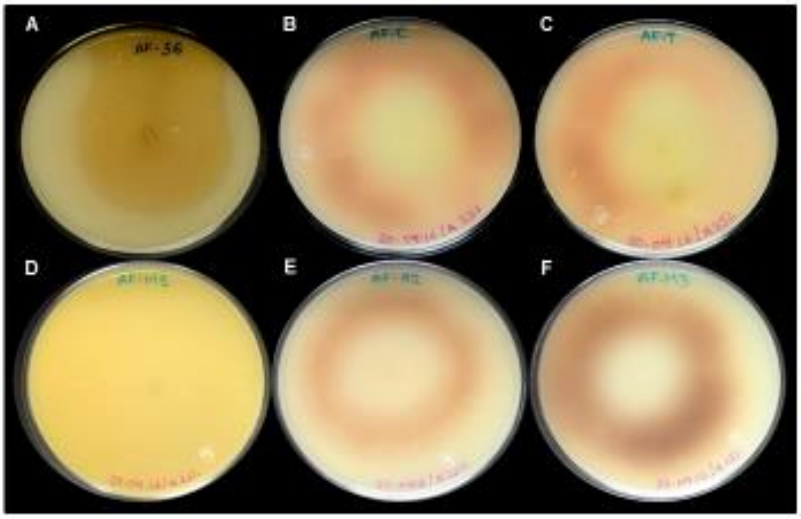
Isolate	Stipe			Vesicle			Spore				Sclerotium			Fialide		
	Mean	LL	UL	Mean	LL	UL	Mean	LL	UL	Mean	LL	UL	Mean	LL	UL
AF-C	237 c	183	291	33.6 c	27.5	39.7	3.3 b	3.1	3.4	418 cd	353	483	5.9 ab	4.9	6.9
AF-T	646 a	564	727	76.6 a	67.7	85.5	2.8 c	2.7	2.9	453 bc	361	545	6.4 ab	4.9	7.8
AF-AC1	313 ab	241	386	54.3 b	46.3	62.2	3.1 b	3.0	3.3	592 ab	516	667	5.1 b	4.4	5.8
AF-AC2	373 ab	292	454	58.2 b	49.3	67.0	3.0 bc	2.8	3.1	428 cd	352	503	5.1 b	4.3	5.8
AF-AC3	441 ab	347	534	47.8 ab	37.5	58.0	3.1 b	2.9	3.3	--	--	--	6.6 ab	5.5	7.7
AF-EM1	331 ab	255	407	50.5 b	42.1	58.8	3.5 a	3.4	3.7	268 d	193	344	4.8 b	4.1	5.5
AF-EM2	218 c	115	320	38.8 ab	27.6	50.1	3.2 b	3.1	3.4	672 a	597	748	5.4 ab	4.1	6.6
AF-EM3	320 b	239	401	47.3 ab	38.4	56.2	3.6 a	3.5	3.8	561 ab	505	617	6.9 a	6.0	7.9

-- Without sclerotium- LL, UL, lower and upper limit of the confidence intervals.

abcd Means of columns with different letters show significant statistical differences ($P<0.05$).

The isolates identified as *A. flavus* were analyzed using TLC and ammonium vapors in coconut agar techniques in order to identify the presence of aflatoxin (Figure 3). Five isolates (AF-AC1, AF-AC2, AF-AC3, AF-EM2 and AF-M3) had a positive reaction to the presence of AF, while the other (AF-EM1) produced no aflatoxins, with both techniques. These results coincided with the amplified aflR gene.

Figure 3: Intensity of color in a coconut agar medium in contact with ammonia vapors of *Aspergillus flavus* isolates after 4 d of growth

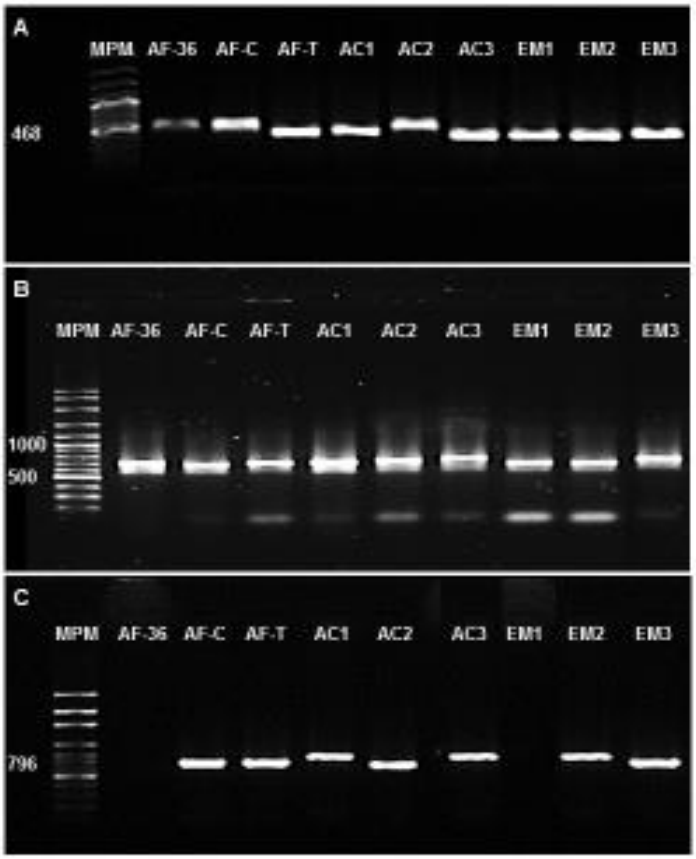


Panels: **A**) AF-36, non-aflatoxigenic (AF-). **B**) AF-Cuatitlán, and **C**) AF-Tamaulipas, aflatoxigenic (AF+). **D**) AF-EM1, AF- isolate from corn silage. **E**) AF-AC2, AF+ isolate from concentrated feed. **F**) AF-EM3, AF+ isolate from corn silage.

Molecular analysis

Of the 88 isolates morphologically identified as *Aspergillus* spp., 49 % were amplified for the calmodulin gene, and 31 % were amplified for ITS (Figure 4). The analysis of the sequences obtained showed that the species were *A. oryzae* (45.5 %), *A. niger* (10.2 %), *A. ochraceus* (3.4 %), *A. pseudodeflectus* (4.5 %), *A. ustus* (10.2 %), *A. flavus* (6.8 %), *A. versicolor* (5.6 %), *A. nidulans* (5.6 %), *A. sublatus* (4.5 %) and *A. Sydowii* (3.4 %).

Figure 4: Electrophoresis in an agarose gel at 1% of the PCR products amplified from the calmodulin gene (Panel A), internal transcribed spacer of the rDNA region 5.8S-ITS2 (Panel B), and regulatory gene of the aflatoxin biosynthetic pathway, aflR (Panel C)



Rails: MWM) Molecular weight marker (100pb). Controls: Non-aflatoxigenic *A. flavus* (AF-36) and aflatoxigenic fungi (Cuautitlan, AF-C; Tamaulipas, AF-T). *A. flavus* isolates of corn silage (EM1, EM2 and EM3) and feed concentrate (AC1, AC2 and AC3) intended for dairy cows

The five local isolates identified as aflatoxigenic *A. flavus* amplified a fragment of 796 pb for the aflR gene. The analysis of the sequences of the control strains (AF-36, AF-C and AF-T) and the six isolates of *A. flavus* (AF-AC1, AF-AC2, AF-AC3, AF-EM1, AF-EM2 and AF-EM3) have been shown to have an identity percentage of over 90% with the isolates of *A. flavu*s registered in the database of the NCBI (Table 3).

Table 3: Identity of the isolates of *Aspergillus flavus* obtained from diets for dairy cows in Aguascalientes, Mexico, compared to existing scripts at the National Center for Biotechnology Information (NCBI)[a]

ID of the Isolate	Origin	Primer[b]	Access code	Coincidence (%)
Control- AF-36	Control	ITS1	LN482513.1	99
		ITS4	KX550912.1	98
Control+ C (AHY255)	Corn (Cuatitlan)	CMDA7F	AY974341.1	98
		CMDA8R	AY974340.1	96
		ITS1	LC105688.1	100
		ITS4	KT254587.1	99
		AFLRF	FN398160.1	100
		AFLRR	L32576.1	100
Control+ T (AHY256)	Corn (Tamaulipas)	ITS1	KX015990.1	100
		ITS4	KF221065.1	99
		AFLRF	XM_002379905.1	100
		AFLRR	AF441435.2	99
AC1 (AHY257)	Feed concentrate	ITS1	KF434090.1	100
		ITS4	KF434090.1	99
		AFLRF	AF441434.1	100
		AFLRR	XM_002379905.1	99
AC2 (AHY258)	Feed concentrate	CMDA7F	AY974341.1	99
		ITS1	KM285408.1	100
		ITS4	EF409812.1	95
		AFLRF	XM_002379905.1	100
		AFLRR	FN398160.1	93
AC3 (AHY259)	Feed concentrate	CMDA7F	XM_002374071.1	99
		CMDA8R	XM_002379905.1	99
		ITS1	KF434090.1	99
		ITS4	KT356196.1	97
		AFLRF	AF441435.2	99
		AFLRR	AF441434.1	99
EM1 (AHY195)	Corn silage	CMDA7F	AY510451.1	95
		CMDA8R	XM_002374071.1	100
		ITS1	HQ010119.1	99
		ITS4	KX641192.1	99
		ITS1	KT356196.1	98

		ITS4	LN482513.1	100
EM2 (AHY203)	Corn silage	AFLRF	MH752566.1	100
		AFLRR	KY769955.1	100
EM3 (AHY204)	Corn silage	CMDA7F	XM_002374071.1	100
		CMDA8R	XM_002374071.1	100
		ITS1	KX572367.1	99
		ITS4	KT356196.1	99
		AFLRF	L32577.1	100
		AFLRR	MH752564.1	100

[a] NCBI (National Center for Biotechnology Information): https://blast.ncbi.nlm.nih.gov/

B CMDA = calmodulin; ITS = internal transcribed spacer; AFLR = regulator of the biosynthetic pathway; R = Reverse; F = forward.

Aflatoxins detected in feed and in milk

Of the 288 samples obtained from food, 286 (99.3 %) exhibited detectable levels of AFs (18.5 ± 3.7 µg/kg); of which 10.4 % exceeded the maximum limits allowed by the Mexican law (20 µg/kg). The presence of AFM_1 (0.021 µg/kg) was detected in 39.9% of the 183 samples of raw milk, and 12.0% exceeded the maximum permissible limit used as a standard by the agro-industry which acquired the raw milk (0,050 µg/kg). The highest incidence of AF in feed occurred in summer and autumn ($P<0.01$), compared to winter and spring (17.4^{a} ± 3.2 and 14.8^{ab} ± 1.6 *vs* 8.1 ^{bc} ± 0.5 and 5.9^{c} ± 0.5 µg/kg, respectively). In turn, the concentration of AFM_1 was directly correlated with the concentration of AFs in the TMR in a double squared model ($P<0.01$; R^2= 30.5 %). Also the presence of AFM_1 in milk was significantly correlated with the level of milk production and the consumption of TMR; on average, the cows consumed daily 621 µg of aflatoxins in 42 kg of TMR; had a daily production of 26.2 L of milk with a total load of 0.603 µg of AFM_1 —which represented a great capacity of dairy cows to metabolize the AF—, and eliminated only a fraction (0.09 %; Table 4); in general, cows with a high production were exposed to the ingestion of larger amounts of AFs (658 µg/cow/day), and the presence and elimination of AFM_1 in the milk of highly productive cows was greater than in cows with a medium and low production (0.22 µg/cow/d).

Table 4: Average exposure (± SE) of dairy cows to the natural contamination by aflatoxins in the total mixed ration (TMR) and average elimination of AFM_1 per productive batch

Batch	Cows	Milk production	TRM	AF ingestion		AFM_1 elimination			
	(No)	(Kg/cow/day)	(Kg/cow/day)	(µg/cow/day)		(µg/kg)		(µg/cow/day)	(%)
High	1760	32.0[a]	44.7[a]	658[a]	± 97.0	0.024[a]	± 0.005	0.72	0.11
Means	606	20.2[b]	36.4[c]	546[a]	± 83.4	0.022[a]	± 0.005	0.44	0.08
Low	388	14.8[c]	38.4[b]	568[a]	± 82.6	0.015[b]	± 0.004	0.22	0.04
Total	2754	28.0	42.0	621	± 92.0	0.021	± 0.005	0.58	0.09

[ab] Different letters indicate significant differences between production batches $P<0.05$.

Discussion

In this two-year longitudinal study, the presence of *Aspergillus flavus* in the feed for dairy cows in the Central High Plateau of Mexico was proven. These isolates exhibited morphological, toxicological and molecular features which are consistent with the strains that have the capacity to produce aflatoxins. Furthermore, the accumulation of AFs in the feed and in the milk produced by cows was corroborated. Although information about the existence in Mexico of indigenous *A. flavus* populations with and without the ability to produce aflatoxins was already available, in general terms, this study adds for the first time the molecular, toxicological and morphometric characterization of *A. flavus* to the quantification of aflatoxins in the dairy, which is relevant for livestock production and public health.

Frequency of *Aspergillus* spp. and *A. flavus*

The fungal genus *Aspergilus* had the highest occurrence at the DPU (31 %), followed by *Penicillium* (13.8 %) and *Fusarium* (12.7 %). These genera have already been identified in Mexico in stored yellow corn[9] and in corn hybrids intended for human and animal consumption[30]. It has been suggested that the frequent presence of micotoxigenic fungi and their toxins in corn is due to improper handling of the crop and to ineffective implementation of strategies for the control of infections[5,31].

In this study, *A. flavus* was isolated from the feed for dairy cows at a ratio of 6.8% of the total identified *Aspergillus* spp. (6/88). This species was not identified in agricultural soil intended for the alternating production of fodder maize and oats. In Mexico, *A. flavus* has been

identified in commercial corn kernels[32] from soil for crops of grain corn[10]. This suggests that fungal populations of this species are distributed across the agricultural areas of Mexico.

In this study, the morphometric analysis showed that one-third of the isolates of *A. flavus* contained abundant short sclerotia (< 400 µm) and were therefore classified as *S* strains, while the rest were classified as *L* strains, as they had few but larger sclerotia (>400 µm). The importance of this morphology and its association with the aflatoxigenicity of S strains has already been pointed out; on the other hand, *L* strains contain both toxigenic and atoxigenic isolates[33,34].

Molecular analysis

All of the 25 sequences obtained from the isolates identified morphologically as *A. flavus* exhibited a large percentage of identity (> 90%) with registered sequences of *A. flavus* strains. Amplified fragments were also obtained for the 468 pb CaM gene, for the 796 pb aflP gene, and for the ITS with a range of 600-800 pb (ITS1-5.8S-ITS2). It has been pointed out that the rDNA region of the internal transcribed spacers is the official DNA bar code for fungi because it is the most frequently sequenced marker and is a useful tool for the description of *A. flavus* species[8]. It has also been said that the CaM gene is able to distinguish between almost all species of *Aspergillus*[8]. On the other hand, the aflR gene is necessary for the transcription of most genes involved in the activation of enzymatic reactions that are necessary for the formation of aflatoxins[35-37]. Based on the above, this work integrates molecular identification to the morphological identification of six isolates of *A. flavus* obtained from the dairy production chain in the Central High Plateau region of Mexico.

Aflatoxigenic capacity

The six isolates identified as *A. flavus* were evaluated with TLC and vapors of ammonium in coconut agar in order to identify their capacity to produce aflatoxins; five were classified as aflatoxigenic fungi, and one was classified as a non-producer of aflatoxins. These results evidenced a behavior comparable to those obtained in other studies conducted in Mexico[10] in which *A. flavus* isolates were identified as genetically related to *A. flavus* AF-36[32]. This non-aflatoxigenic strain has been employed in the development of biological control strategies to reduce aflatoxin production of aflatoxigenic strains in cotton-producing fields[15]. The above suggests that the main aflatoxigenic *A. flavu*s populations are distributed worldwide across agricultural ecosystems, and that they coexist with strains that have no capacity to produce aflatoxins.

Aflatoxins detected in feed and in milk

A total of 99.3 % (286/288) of the samples of feed for dairy cows had detectable levels of AFs (18.5 ± 3.7 µg/kg), of which 10.4% exceeded the maximum limits allowed by Mexican law (20 µg/kg); furthermore, 39.9 % (73/183) of the raw milk samples exhibited the presence of AFM_1. Comparable proportions of AF contamination have been detected in balanced feeds produced in Mexico[5], in the total mixed rations of stables in Mexico[38], and in samples of feed of Asian origin[39]. The incidence of AFM_1 has been reported in raw bovine milk in Mexico[6,7]; also, incidence of AFM_1 has been identified in milk for human consumption in various countries[3,40-42]. This suggests that the aflatoxin contamination of food for human and animal consumption is a global public health issue.

This study showed an association between the level of milk production of cows with the amount and concentration of AFM_1 eliminated in the milk, in such a way that highly productive cows consumed a larger amount of feed and were exposed to larger amounts of aflatoxins in their feed; therefore, the total amount was larger, and the concentration of AFM_1 in raw milk was higher ($P<0.05$). It has been pointed out that the AFM_1 elimination rate in milk is influenced by the amount of feed consumed per day and the amount of milk produced per day by each animal, as well as by the stage of lactation[43].

Conclusions and implications

This study proved morphologically, toxicologically and molecularly the occurrence of *A. flavus* in corn silage and in the total mixed ration, which led to the aflatoxin contamination of almost all diets of dairy cows, as well as to the residual presence of AFM_1 in raw milk. Dairy cows were able to metabolize and eliminate more than 99 % of aflatoxin present in their diet through routes other than milk.

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Conflicts of Interest

The authors declare that there is no potential conflict of interests with respect to the present research or to the authorship and/or to publication of this article.

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