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Molecular Characterization, Toxicogenic Potential and Antifungals of Clinical and Environmental Isolates of *Aspergillus flavus* in Aspergillosis

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ABSTRACT

The identification of clinical and environmental Aspergillus species in human aspergillosis and the production of aflatoxin B1 inside the body is important as well as to discover which fungicide can control it.

Twelve clinical human aspergillosis cases and 14 environmental isolates were identified by morphological characteristics. DNA extraction, purification, amplification, and sequencing of the clinical human aspergillosis, the environmental isolates were carried out and quantified by HPLC, to determine the AF toxicogenic potential of each fungal strain and their susceptibility to antifungals. Phylogenetic analysis was performed on the Aspergillus isolates. A data base was created in Microsoft Excel® Office Professional Plus 2013 and was imported into the statistical program Minitab®19 version 19.1.

Aspergillus flavus was present in 7/12 cases of clinical human aspergillosis ($\bar{x}$ AFB1= 2227.7 ng mL-1) and 6/14 environmental isolates ($\bar{x}$ AFB1= 396.2 ng mL-1). Coconut agar medium stimulated AFB1 production. All isolates were sensible to µg of ketoconazole and no isolates were sensitive to 25 µg of fluconazole.

The conclusion is that A. flavus was found in the human aspergillosis samples. More AFB1 was produced by the clinical samples than the environmental samples, and all isolates were susceptible to 50 µg of ketoconazole.

Keywords
Aspergillosis, Aflatoxin, Phylogenetic analysis, ketoconazole, Aflatoxigenic potential.

Introduction
In 1729, Micheli gave the first description of the genus *Aspergillus,* and in 1809, Link validated this description according to the International Code of Botanical Nomenclature [1,2]. Molecular techniques are increasingly being used to complement macroscopic

and microscopic examinations, particularly internal transcribed spacers (ITSs) of rDNA [3,4].

Aflatoxins (AFs) are secondary fungal metabolites, chemically bis dihidro furan coumarins, they are the most common and toxic mycotoxins produced mainly by three fungal species, *Aspergillus flavus, A. parasiticus* and *A. nomius. Aspergillus flavus* is used industrially and has toxigenic potential [5,6]. AFs act on DNA by a genotoxic mechanism that requires a metabolic conversion to the reactive (electrophilic) form to transform into a mutagenic adduct that accumulates and produces damaging effects [7,8].

AFs are synthesized during the stationary phase of fungi and are associated with the differentiation and sporulation of the fungus at the end of the mycelial growth phase. AF synthesis occurs in the hyphae, conidia, mycelium, biomass and sclerotia produced by the fungus [7,8].

There are different types of AFs, among which AFB1 is the most carcinogenic secondary metabolite produced by *A. flavus* and related species that contaminate agricultural products. AFB1 causes chromosomal aberrations, DNA breakdown in plant and animal cells, and genomic mutations that can be identified by the Ames test [8,9].

The environmental factors that affect the development of aflatoxigenic fungi and the production of AF are: a) Substrate, environmental conditions and physiological responses of fungi, b) Temperature, c) Water activity and relative humidity, d) pH, e) Light and darkness and f) Atmosphere and oxygen concentrations [10,11].

A. flavus, with aflatoxigenic potential, is the second leading cause of localized and systemic infections in the clinic, mainly affecting immunocompromised patients. Its resistance to azoles is currently a concern because these drugs are among the first choices for the treatment of *A. flavus* infections [4,12].

Methodology

The present research was carried out in the Virology and Mycology Research Department of the National Institute of Respiratory Diseases "Ismael Cosío Villegas" (Departamento de Investigación en Virología y Micología, Instituto Nacional de Enfermedades Respiratorias, “Ismael Cosío Villegas”, INER), in the Mycotoxin Laboratory of the Institute of Biology of the National Autonomous University of Mexico, (UNAM), and in the Children's Hospital of Mexico "Federico Gómez" (Hospital Infantil de México “Federico Gómez”), which provided the samples.

The human samples were taken from sputum or saliva that was cultivated and the morphological and molecular identification of the fungi were done. The environmental samples were taken with hyssop in surfaces of the patient bathroom, balustrade or railing where the patient was. Fungi were present in clinical and environmental aspergillosis samples in Mexico City. These fungi were identified and verified, their aflatoxigenic potential was analyzed, and their susceptibility profile to antifungals was determined.

The completeness of the current database available for each gene to be analyzed and selection of the phylogenetic analysis to estimate their evolutionary distance are considerations when identifying fungi. There are three molecular barcodes that were used: calmodulin (*CaM*), β-tubulin (*BenA*) and the second largest subunit of RNA polymerase II (RPB2) [13,14].

Sample size and Sampling

Twelve clinical *Aspergillus* isolates (CI 01-11 and 13) and fourteen hospital environmental isolates (EI 01-14) were provided by the Infectology Laboratory of the "Federico Gómez" Children's Hospital of Mexico City. The origins of each sample are described in Table 1. In addition, an aspergillosis clinical isolate (CI-12) from sputum was obtained from the INER.

The human samples were taken from sputum or saliva that was cultivated and the morphological and molecular identification of the fungi were done. The environmental samples were taken with hyssop in surfaces of the patient bathroom, balustrade or railing where the patient was. All fungal samples were preserved by sand desiccation. Each sample was reactivated in triplicate, in Petri dishes with potato dextrose agar (PDA, BD Bioxon, Franklin Lakes, USA) and incubated for 7 days at 25 °C ± 2 °C. All cultures were identified by their morphological, macroscopic and microscopic characteristics.

DNA extraction and Purification

For identification at the species level, DNA was extracted from each positive growing culture with a DNA purification kit (Wizard Genomic DNA Purification, Madison WI, USA) using the manufacturer's protocol with a lysis modification. The lysis modification was performed with 600 µL of Nuclei Lysis Solution and 0.2 g of sterile glass beads, and all cultivated fungal mycelia were transferred to Fast Prep-24™ 5G tubes (MP Biomedicals, Santa Ana, California, USA).The mycelia mixed with lysis buffer and glass beads were homogenized for 4 cycles of 40 sec each at a speed of 6 m/s, placing the tubes in ice for 5 min between each cycle. The remaining steps were performed according to the manufacturer’s instructions until DNA was obtained.

The DNA was quantified with a spectrophotometer (Nano Drop 2000, Thermo Fisher Scientific, Wilmington, DE, USA), and its concentration was adjusted to 10 ng μL^{-1} for the amplification reaction.

Fungal DNA amplification reaction and sequencing

The amplification reaction was performed with DNA (10 ng μL^{-1}) Master Mix KAPA SYBR FAST qPCR (2X) (Kapa Biosystems, Cape Town, South Africa) and primers, including β-tubulin (5’-TGACGGGTGATTGGGATCTC-3’ and 5’-CGTCCGCTTCTT CC TTGTTT-3’), using one initial

denaturation cycle of 95°C for 3 min, followed by 25 cycles of 95 °C for 20 sec, 60°C for 15 sec, and 72°C for 20 sec and a final extension at 72 °C for 3 min. The products were purified and concentrated to 9 µL with a Zymo Research Concentrator kit (Zymo Research, Irvine, California USA) according to the manufacturer´s instructions. The purified products were mixed with the sequencing BigDye® Terminator v3.1 reaction mix (Life Technologies, Carlsbad, CA, USA). The sequences were obtained by capillary electrophoresis with a genetic analyzer ABI Prism 3730, Life Technologies).

Molecular identification of *A. flavus* strains

The sequences were assembled using BioEdit V7.0.5.3 and MEGA 11 and found in GenBank (MN449963-MN 449975). The β-tubulin sequences of the clinical and hospital environmental samples were aligned using Clustal W with other *Aspergillus* species belonging to the Flavi section (*A. flavus, A. parasiticus, A. nomius, A. pseudotamarii* and *A. fumigatus* were used as outgroups), and subsequently, phylogenetic analysis was performed with MEGA 11 using the neighbor-joining method with 1000 iterations and determining the evolutionary distance with the Kimura 2-parameter model.

Aflatoxigenic potential of the clinical and environmental strains of *A. flavus*

AF-extracted conidia were recovered from the reactivated clinical and environmental samples, in PDA Petri dishes after the incubation period; and the surface of the culture was washed with sterile water (PiSA). Conidia were quantified with a Neubauer chamber (MarienFeld, Germany) and brought to a concentration of 1×10^5 conidia mL^{-1} to inoculate duplicate Petri dishes with modified coconut agar media (CAM) and yeast extract with sucrose (YES). CAM contained 250 mL of coconut milk (GoldenStar®), 250 mL of distilled water and 9 g of agar, and YES contained 10 g of yeast extract (Sigma®), 100 g of sucrose (BD®) and 8 g of agar (BD®) in 500 mL of distilled water.

The media, inoculated in the center of the Petri dish, were incubated at 26 °C for 7 days. At the end of this time, the reverse of each culture was observed with UV light (360-365 nm), and the presence of fluorescence was sought in the contour of the developed colonies. All samples that fluoresced received 70 mL of HPLC-grade MeOH (J.T. Baker, USA), and the total mixture was transferred to a blender that was operated at maximum speed for 2 min (Waring 2-speed Lab Blender). The liquefied samples were transferred to Falcon tubes to be centrifuged at 2200 rpm for 5 min, and the organic layer was decanted and filtered through Whatman # 4 paper. The total filtrate of each sample was evaporated in a biosafety cabinet (Telstar Bio IIA) for 72 h, and the resulting extracts were resuspended in extraction solvent and analyzed.

The qualitative detection of AFs was carried out by thin layer chromatography (TLC) on 20 × 20 cm plates that were 0.25 mm thick (TLC Silica gel 60 Merck Darmstadt, Germany). Each 30 µL sample was spotted using a 2 cm micropipette from one point to another, and a 1000 ng mL^{-1} AFB1 standard of was used as a reference. The plates were developed for 20 min with a mobile phase consisting of acetone-chloroform (90:10 v/v). The plates were visualized under UV light (360-365 nm).

AFB1 production and HPLC analysis

The extraction of AFB1 was performed according to the R-Biopharm method [15], which was recommended for use with the Easi-Extract aflatoxin immunoaffinity column (IAC). An IAC was equilibrated with 20 mL of PBS (pH 7.4) applied at a flow rate of 5 mL^{-1}. Each sample was added independently to a new column. AFB1 was eluted using 1.5 mL of HPLC-grade MeOH followed by 1.5 mL of distilled water under reflux conditions. The eluate was dried in a furnace at 40 °C (Model F135A, Novatech®) and then derivatized with a trifluoroacetic acid solution as described [16,17].

The contents were evaporated to dryness, and the samples were resuspended in 1 mL of HPLC-grade MeOH. Sixty microliters of the mixture was injected into an Agilent Series 1200 HPLC system (Agilent Technologies, Inc., USA) consisting of an isocratic pump (Model G1310A), a fluorescence detector (Model G1310A Series DE62957044, Agilent Technologies, Inc., USA) set to an excitation wavelength in the range of 360 to 362 nm with a maximum emission of 425 nm, and an automatic injector (G1329A SERIES DE64761666). The chromatographic column was a VDS Optilab VD Spher 100 C18–E (5 µm, 250 × 4.6 mm) (Berlin, Germany) maintained at room temperature (22 °C) with a mobile water phase of CAM/methanol/distilled water (65:15:20 v/v/v), which was degassed for 30 min by vacuum filtration; the samples were eluted at a flow rate of 1.0 mL min^{-1}. Calibration curves were constructed using eleven concentrations of the AFB1 standard: 2, 5, 10, 20, 50, 100, 200, 400, 600, 800 and 1000 ng mL^{-1}. The HPLC quantification method followed that previously described [18].

In vitro Susceptibility to Antifungals

The susceptibility test that was used in this study is an adaptation of the M-51A technique "Method for the test of sensitivity to the diffusion of antifungal discs of non-dermatophyte filamentous fungi" standardized and approved by the Clinical Laboratory Standards Institute (CLSI) with the Kirby-Bauer method [19]. The conidia were recovered by washing the PDA surface with sterile water (PiSA®), placed in a Neubauer chamber (MarienFeld®, Germany) and diluted to approximately 1.5×10^3 mL^{-1}. Fifty microliters of this suspension was inoculated into the center of a Petri box of Dextrose Sabouraud Agar culture medium (BD Bioxon®) by making a very large stretch mark on the entire surface of the agar. Subsequently, the discs were placed in duplicate for antifungal activity determinations with 100 µg of amphotericin B, 50 µg of ketoconazole, and 25 µg of fluconazole (Bio-Rad®) for 72 hours of incubation at 26 °C.

Statistical analysis

A database was created in Microsoft Excel® Office Professional

Plus 2013, into which the information obtained was tabulated. Subsequently, the database was exported to the statistical program Minitab® 19 version 19.1, where analysis of the results obtained throughout this research was carried out.

Results

Origins of the clinical and environmental isolates

In all cases, *A. flavus* was the etiological agent from all the cultures with fungal growth that were amplified with β-tubulin primer (Table 1). The etiological agent of Sample C12 was also *A. flavus* according to its analyzed sequence. In human samples, sex refers to a set of biological attributes that are associated with physical and physiological features.

The data that support the findings of this study are available from the Gene Bank, NCBI. These sequence were found in the Gene Bank with the access MN449963, MN449964, MN449965, MN449966, MN449967, MN449968, MN449969, MN449970, MN449971, MN449972, MN449973, MN449974 and MN449975.

Macromorphological characteristics of *A. flavus*

All cultures had morphological characteristics, both macroscopic and microscopic, that were compatible with *A. flavus*.

Phylogenetic analysis

We verified the identity of all samples by using the reference sequences and constructing a phylogenetic tree (Figure 1). The

Table 1: Characteristics of clinical and environmental *Aspergillus flavus* isolates as etiological agent.

Isolated		Origin	Place of origin	Year of isolation	GenBank Access	Etiological agent
Environmental	A3	HE	FGCHM	2007	MN449970	*A. flavus*
	A8	HE	FGCHM	2008	MN449971	*A. flavus*
	A9	HE	FGCHM	2007	MN449972	*A. flavus*
	A10	HE	FGCHM	2008	MN449973	*A.flavus*
	A11	HE	FGCHM	2008	MN449974	*A. flavus*
	A12	HE	FGCHM	2008	MN449975	*A. flavus*
	TCCA®	Moldy corn	Iowa, USA	----	----	
Clinical	C2	Paranasal sinus	FGCHM	2014	MN449963	*A. flavus*
	C3	Maxillary turbinate	FGCHM	2014	MN449964	*A. flavus*
	C6	Nose	FGCHM	2015	MN449965	*A. flavus*
	C8	Bronchial aspiration	FGCHM	2016	MN449966	*A. flavus*
	C10	Lung	FGCHM	2018	MN449967	*A .flavus*
	C11	Bronchoalveolar lavage	FGCHM	2019	MN449968	*A. flavus*
	C12	Bronchoalveolar lavage	INER	2019	MN449969	*A. flavus*

HE: Hospital Environment; FGCHM: "Federico Gómez" Children's Hospital of Mexico; INER: National Institute of Respiratory Diseases "Ismael Cosío Villegas".

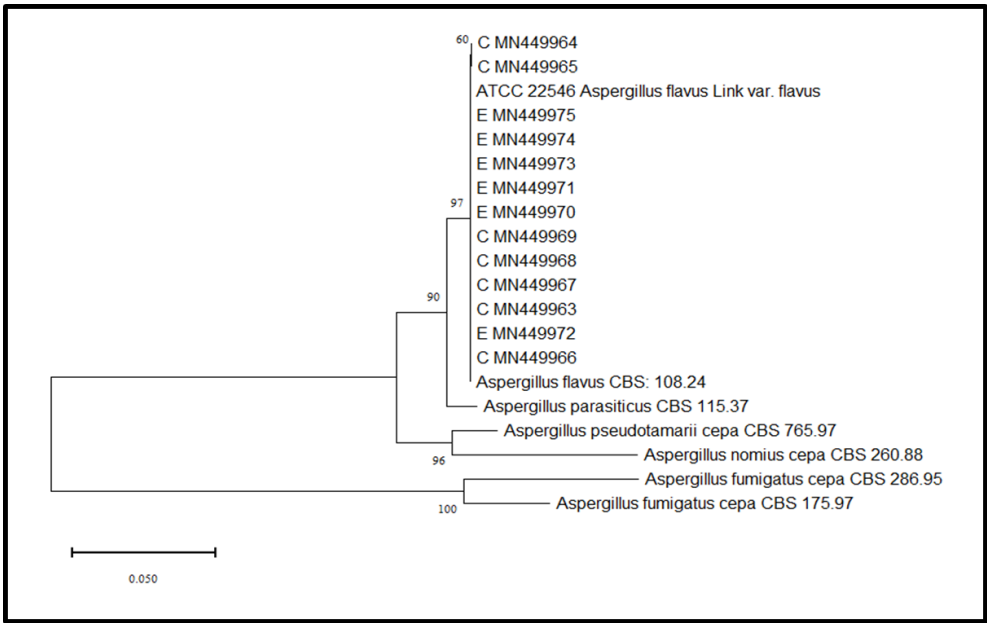

Figure 1: Phylogenetic tree of clinical (C) and environmental (E) isolates with GenBank access compared with other *Aspergillus* species belonging to section *Flavi* (*A. flavus* CBS 108.24, *A. parasiticus* CBS 115.37, *A. nomius* CBS 260.88, *A. pseudotamarii* CBS 765.97) in addition to *A. fumigatus* CBS 175.97 and 286.95 as outgroup.

recovered fungal isolates from 13 samples (7 clinical and 6 environmental) were identified as *A. flavus*. All samples in our study, regardless of their environmental or clinical aspergillosis origin, were completely identical in sequence and made a single branch that included the ATCC strain and the *A. flavus* strain used as a reference. The other reference sequences, including *A. fumigatus*, formed clades distinct from *A. flavus* and allowed us to validate the identity of the analyzed microorganisms.

Aflatoxigenic potential

The qualitative AFB1 production results obtained by TLC from differential culture media (CAM and YES) are shown in Table 2.

Table 2: Thin Layer Chromatography (TLC) Results.

Isolates		Differential culture media	
		CAM	YES
Environmental	A3	-	-
	A8	-	-
	A9	+	+
	A10	-	-
	A11	-	-
	A12	-	+
	ATCC®	+	+
Clinic	C2	-	-
	C3	+	+
	C6	+	+
	C8	+	-
	C10	+	+
	C11	-	+
	C12	-	+

CAM: modified coconut agar medium; YES: Yeast and sucrose extract, negative; + positive.
TCCA® Control moldy corn from Iowa, USA

Table 2 also shows the concentrations of AFB1 produced by clinical aspergillosis isolates and environmental isolates quantified by HPLC. More AFB1 was produced by the clinical isolates ($\bar{x}$ = 2227.7 ng mL^{-1}) than by the environmental isolates ($\bar{x}$ = 396.2 ng mL^{-1}). These data show the relationship between AFB1 and aspergillosis.

The CAM was selected for HPLC quantification because it stimulated the isolates to produce a greater amount of AFB1, as visualized under UV light (Figure 2).

High-Performance Liquid Chromatography (HPLC)

The constructed AFB1 calibration curve displayed good linearity with a correlation coefficient (R^2) of 0.9949 are shown in Figure 3.

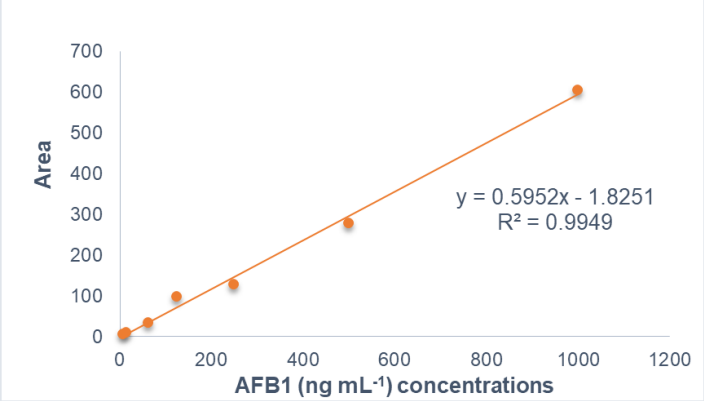

Figure 3: Calibration curve presenting different concentrations of the AFB1 standard.

The chromatograms of the AFB1 standard at a concentration of 250 ng $mL^{-1}$and the AFB1 produced from an environmental isolate and a clinical isolate all had a peak with a retention time of approximately 8.3 min (Figure 4).

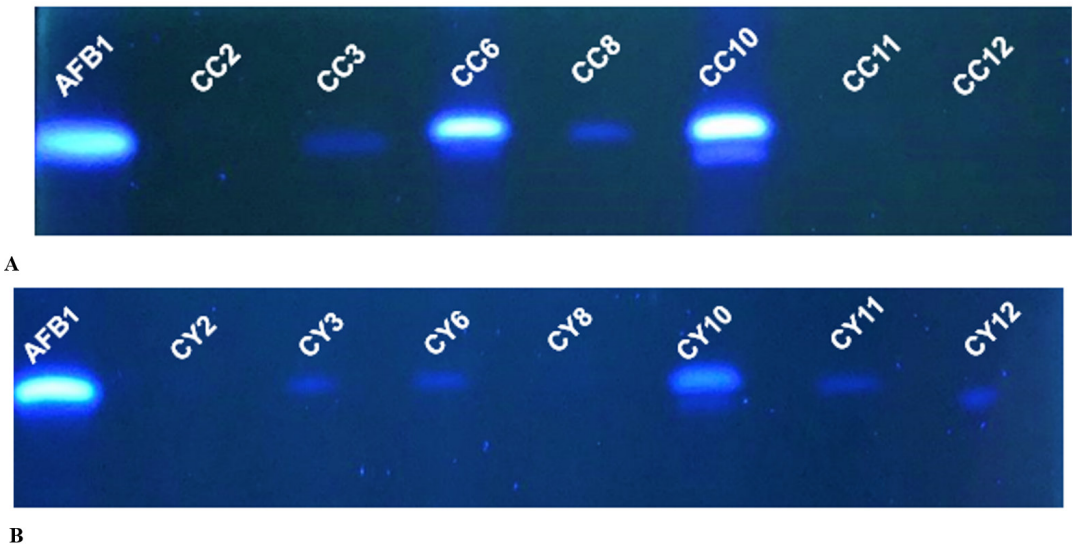

Figure 2A: Comparison of AFB1 production from clinical isolates in coconut agar media (CAM) and yeast extract with sucrose (YES) agar media by thin layer chromatography. A = Clinical isolates with CAM (CC). B = Clinical isolates with YES (CY). **B:** Comparison of susceptibility to Amphotericin B (100 µg) of clinical and environmental isolates.

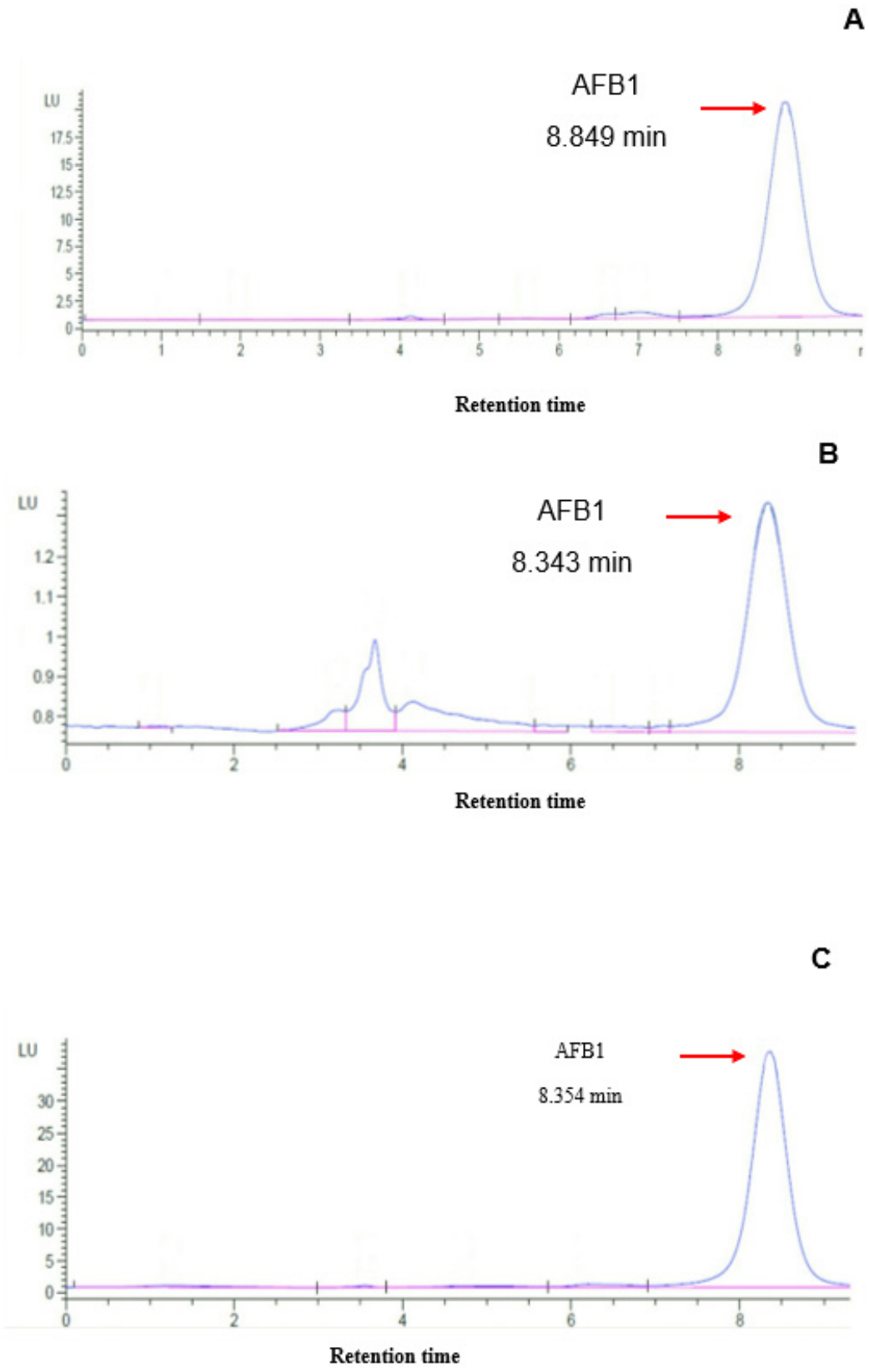

Figure 4: Calibration curve presenting different concentrations of the AFB_1 (Retention times 8.343, 8.354, 8.849 min). (A)

AFB1 standard control, (B) Environmental sample and (C) Clinical sample.

The results obtained in the antifungal susceptibility tests are shown in Table 3.

Table 3: Quantification of AFB_1 in environmental and clinical isolates obtained by HPLC.

Isolates		AFB_1 (ng mL^{-1}) ± SD
Environmental	A3	26.06 ± 0.66
	A8	19.31 ± 2.12
	A9	66.58 ± 1.20
	A10	38.01 ± 0.30
	A11	63.80 ± 1.62
	A12	18.14 ± 0.20
	ATCC®	2541.41 ± 137.32
Clinical	C2	8.54 ± 0.00
	C3	2156.76 ± 7.35
	C6	5955.79 ± 0.00
	C8	854.29 ± 43.91
	C10	6592.68 ± 0.00
	C11	11.97 ± 0.07
	C12	14.02 ± 0.21

TCCA® Control moldy corn from Iowa, USA.

According to the data, 100% of the clinical aspergillosis isolates and environmental isolates demonstrated sensitivity to ketoconazole (50 µg), Table 4.

Table 4: Susceptibility profile to antifungals.

	Antifungic		
	AB 100 µg (ratio mm)	**KET 50 µg (ratio mm)**	**FCA 25µg (ratio mm)**
A3	-	29	-
A8	9	35	-
A9	10	31	-
A10	12	29	-
A11	12	31	-
A12	-	30	-
ATCC®	12	32	-
C2	-	30	-
C3	9	25	-
C6	8	28	-
C8	-	26	-
C10	8	27	-
C11	11	30	-
C12	-	28	-

AB = Amphotericin B. KET = Ketoconazole. FCA = Fluconazole. Resistant < 10 mm. Intermediate 10 mm – 20 mm. Sensitive > 20mm.

The clinical isolates produced an inhibition halo that was smaller in diameter ($\bar{x}$= 27.7 mm) than the environmental isolates ($\bar{x}$= 31 mm). In relation to the antifungal amphotericin B (100µg),85.7% of the clinical isolates showed resistance and 14.3% showed intermediate resistance, while 57.1% and 42.9% of the environmental isolates showed resistance and intermediate resistance, respectively (Figure 5). None of the clinical or environmental isolates showed sensitivity to fluconazole (25µg).

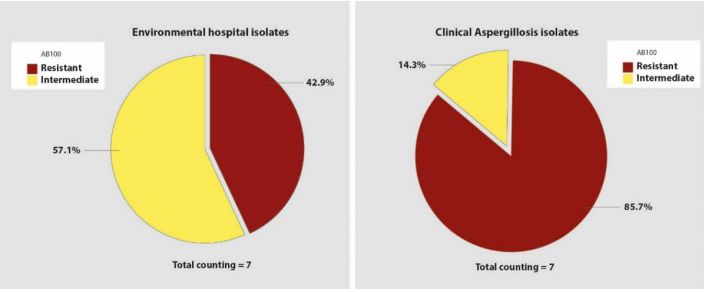

Figure 5: Comparison of susceptibility to Amphotericin B (100 µg) of clinical and environmental isolates.

Discussion

The accurate identification of fungi by their microscopic and macroscopic characteristics is limited to analysis of the spore-producing bodies and their vegetative structures; however, this approach to diagnosing fungal diseases can lead to misidentification [14]. We used an identification and verification approach based on genetic marker (β-tubulin) sequencing and phylogenetic analysis. *A. flavus* was identified and verified in all samples of both environmental and clinical origin. This identification approach can be added to fungal diagnostics and in the future help to identify fungal infections, for example, those associated with COVID-19 [20,21].

A. fumigatus, followed by *A. flavus,* is associated with a wide spectrum of diseases worldwide [4,22]. In Mexico, a study declared that *A. flavus* is the fungus most frequently related to cases of keratitis [23]. *A. flavus* was identified and verified in all of the clinical and environmental samples in this study, and we corroborate that all fungi produced AFB1. The most AFB1 was produced by the clinical isolates in a proportion six times higher than the production of AFB1 from the environmental samples. This may be due to differences in pH, temperature, water activity, and other conditions that the environmental fungi were subjected to compared to fungi that were housed by human hosts. The differences in AFB1 production found in the different types of samples opens the possibility of conducting a more extensive study to determine whether patients with AFB1 are at risk of developing cancer or bleeding conditions.

Although the *A. flavus* in the study were isolated under different conditions (environmental and clinical), they were shown to be susceptible to ketoconazole, and since most samples came from a children's hospital, this drug should be the first choice for treatment. We also tested the susceptibility of *A. flavus* to amphotericin B, and it showed resistance. These results should be considered during treatment in contrast to other reports that recommend avoiding the use of this drug [4,22,24]. Finally, we tested fluconazole on all *A. flavus* strains, and no isolate showed sensitivity; therefore, this antifungal cannot eliminate this fungus and should not be used in patients. The amount of AFB1 produced was not related to the

susceptibility to the antifungals we tested.

In conclusion, for the first time, the present study shows the presence of *A. flavus* both in environmental samples from hospitals and in clinical aspergillosis samples from children in Mexico. The clinical aspergillosis samples produced more AFB1 than the environmental samples, and all isolates were susceptible to ketoconazole. The present research shows the importance of *A. flavus* and the production of aflatoxin B1 in Aspergillosis and its possible control.

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