



## Original Article

# Species distribution and antifungal susceptibility patterns of *Aspergillus* isolates from clinical specimens and soil samples in Mexico

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## Abstract

This study aimed to assess the species distribution and antifungal susceptibility patterns of 200 strains of *Aspergillus* isolated from clinical specimens ( $n = 146$ ) and soil samples ( $n = 54$ ) in Mexico. ITS,  $\beta$ -tubulin, and calmodulin DNA sequencing was performed for species identification. Broth microdilution susceptibility testing for amphotericin B, voriconazole, posaconazole, itraconazole, isavuconazole, anidulafungin, caspofungin, and micafungin was done according to CLSI for all strains. *A. fumigatus* was most frequently recovered from clinical specimens, while *A. niger* was commonly encountered in soil, both followed by *A. flavus* in the second place. A total of 60 (30%) cryptic species were identified, with *A. tubingensis* and *A. tamarii* being the most commonly found. The decreased susceptibility to amphotericin B and azoles was 32% for both, and were mainly led by *A. fumigatus*, whereas this percentage decreased to 9% for caspofungin, particularly in *A. terreus*. More than 75% of cryptic species were susceptible *in vitro* to all antifungals. Multi-azole decreased susceptibility was detected only in seven isolates. Given that antifungal resistance in *Aspergillus* spp. is an increasing worldwide threat that causes major challenges in the clinical management of aspergillosis, these data highlight the need for continuous epidemiological surveillance of these pathogens for the implementation of locally adequate treatment strategies.

## Lay summary

This is an epidemiological study in Mexico. *A. fumigatus* was most frequent in clinical specimens and *A. niger* in soil samples. *A. tubingensis* and *A. tamarii* were the most common cryptic species. Resistance to amphotericin B and azoles was 32% each, and 9% for caspofungin.

**Key words:** *Aspergillus*, cryptic species, molecular epidemiology, antifungal susceptibility, Mexico.

## Introduction

*Aspergillus* species are saprophytic molds widely distributed throughout the environment.<sup>1</sup> These opportunistic fungi can cause a variety of diseases, from allergies to invasive aspergillosis (IA), especially in immunocompromised patients.<sup>2</sup> *Aspergillus fumigatus* remains the main pathogenic species; however, new or rare species are continuously being recognized as etiological agents of human infections.<sup>3,4</sup>

Resistance to antifungal drugs such as azoles and echinocandins and the emergence of cryptic species of *Aspergillus* with variable susceptibility to current antifungals has also appeared worldwide endangering the advances reached in the treatment of this severe fungal infection.<sup>5–9</sup> The clinical implications of azole resistance are just recently being studied, suggesting treatment failure.<sup>10</sup> These reports impelled the need to watch over the frequency of these isolates and their clinical implications.

In Mexico, little is known about the epidemiology of aspergillosis, but it has been estimated that each year at least 4510 patients are affected with IA related to chronic obstructive pulmonary disease, and 506 cases are associated with hematologic malignancies.<sup>11</sup> Azole resistance in *Aspergillus* has been recently reported in Mexico from a small sample of clinical isolates,<sup>12</sup> making the need for precise species identification and continuous antifungal surveillance urgent. In this context, the present study aimed to assess the species distribution and antifungal susceptibility patterns of 200 strains of *Aspergillus* isolated from clinical specimens and soil samples in Mexico.

## Methods

### Ethics statement

This study was evaluated and approved by the local Ethics Committee of the Universidad Autónoma de Nuevo León (registration number: MB19-00004) and was conducted in agreement with Good Laboratory Practices. The committee waived informed consent because no intervention was involved and no patients' identifying information was included.

### Clinical isolates

A total of 146 isolates of *Aspergillus* spp. obtained from different patients admitted to the Hospital Universitario "Dr. José E. González" (Universidad Autónoma de Nuevo León) in Monterrey, Mexico between 2006 and 2019 were included in this study. The isolates were sent for further identification and antifungal susceptibility testing to our reference laboratory. All isolates were identified to the section or species-complex level by morphological examination and were stored as conidial suspensions in sterile distilled water at room temperature until analysis. Eighty-eight percent of the isolates were cultured from respiratory samples: bronchoalveolar lavage ( $n = 47$ ), sputum ( $n = 46$ ),

tracheal aspirate ( $n = 18$ ), nasal tissue ( $n = 9$ ), and others ( $n = 9$ ) (Supplementary Table 1).

### Soil sampling and analysis

From April 2019 to November 2019, soil samples of 0–5 cm depth were collected in sterile plastic bags from multiple sampling sites in public gardens and recreational parks of the metropolitan area of Monterrey, Nuevo León, Mexico. Each sample (~2 g) was suspended in 8 ml of sterile saline with 0.1% Tween 80, vortexed, and allowed to settle. Then, 100  $\mu$ l of the suspension was spread in duplicate onto Sabouraud-dextrose agar supplemented with 50 mg/l chloramphenicol (DIBICO; Cuautitlán Izcalli, Mexico). Plates were incubated at 30°C for 3–5 days and fungal colonies morphologically suggestive of *Aspergillus* were selected, isolated, and stored in sterile distilled water until analysis.

### Morphological examination

The preliminary identification of all fungal isolates was done according to macro and microscopic characteristics following the recommended criteria for *Aspergillus*.<sup>13,14</sup> Colony morphology was determined after 5–7 days of incubation at 30°C on potato-dextrose agar (PDA) (Difco, Detroit, MI, USA). In parallel, micromorphology observations were carried out on slide mounts prepared with lactophenol cotton blue from PDA colonies.

### Molecular identification & phylogenetics

Genomic DNA was extracted from PDA cultures after 7 days of incubation at 30°C using the Quick-DNA Fungal/Bacterial Miniprep® Kit (Zymo Research, USA) and the Disruptor Genie® Instrument (Scientific Industries, USA), following the manufacturer's instructions. The DNA concentration and purity were determined by optical density at 260 nm (OD<sub>260</sub>) and ratios of OD<sub>260/280</sub> and OD<sub>260/230</sub>, respectively. The amplification targeted three informative genetic markers, comprising: the internal transcribed spacer (ITS) region of the rRNA, using ITS1/ITS2 primers;<sup>15</sup> a fragment of the  $\beta$ -tubulin gene (*BenA*) using Bt2a/Bt2b primers,<sup>16</sup> and a portion of the calmodulin gene (*CaM*) using Cmd5/Cmd6 primers.<sup>17</sup> All the primer sets were synthesized by Integrated DNA Technologies (IDT, Inc., USA). The reactions were performed with PCR master mix (Promega, Madison, WI, USA) in a T100 Thermal Cycler (BIO-RAD; Hercules, CA, USA). Once PCR products were purified using the Wizard SV Gel and PCR Clean-Up® Kit (Promega; Madison, USA), they were sequenced by Sanger in an ABI 3130 (Applied Biosystems, Foster City, CA, USA) automatic sequencer according to the manufacturer's specifications. Obtained sequences were assembled and edited with Sequencher v. 5.4.6 (Ann Arbor, MI, USA). Species identifications were assessed by pairwise sequence comparisons with deposits available in GenBank

**Table 1.** Antifungal susceptibilities of 140 non-cryptic *Aspergillus* spp.

Species ( <i>n</i> )	Antifungal agent							
	AMB	VRC	POS	ITC	ISA	ANF	CSF	MCF
<b><i>A. fumigatus</i> (49)</b>								
GM	3.24	0.43	0.16	0.37	0.42	0.03	0.12	0.03
MIC <sub>50</sub> /MIC <sub>90</sub>	4/8	0.5/1	0.25	0.5/1	0.5	0.03/0.13	0.13/0.25	0.03/0.25
Range	1-16	0.13-8	0.02-2	0.06-2	0.13-4	≤0.008-0.25	0.03-1	≤0.008-0.5
%WT/%NWT	38.8/61.2	93.9/6.1	89.8/10.2	91.8/8.2	93.9/6.1		98/2	
<b><i>A. flavus</i> (36)</b>								
GM	2.83	0.96	0.24	0.38	0.56	0.03	0.08	0.05
MIC <sub>50</sub> /MIC <sub>90</sub>	2/4	1/2	0.25/0.5	0.5/1	0.5/1	0.03/0.25	0.06/0.13	0.03/0.25
Range	1-16	0.25-8	0.02-2	0.06-2	0.25-2	≤0.008-0.5	0.03-0.5	≤0.008-0.5
%WT/%NWT	91.7/8.3	91.7/8.3	88.9/11.1	97.2/2.8	88.9/11.1		100/0	
<b><i>A. terreus</i> (27)</b>								
GM	3.61	1.33	0.21	0.42	0.57	0.02	0.15	0.03
MIC <sub>50</sub> /MIC <sub>90</sub>	2/16	1/4	0.25/1	0.25/2	0.5/1	0.01/0.06	0.13/0.25	0.03/0.13
Range	1-16	0.25-16	0.02-2	0.06-16	0.25-4	≤0.008-0.25	0.03-0.5	≤0.008-4
%WT/%NWT	66.7/33.3	74.1/25.9	88.9/11.1	88.9/11.1	88.9/11.1		55.6/44.4	
<b><i>A. niger</i> (27)</b>								
GM	1	0.54	0.24	0.84	0.70	0.02	0.05	0.02
MIC <sub>50</sub> /MIC <sub>90</sub>	1/2	0.5/1	0.25/1	0.5/4	0.5/2	0.02/0.06	0.06/0.13	0.02/0.13
Range	0.25-16	0.25-4	0.02-1	0.13-8	0.25-4	≤0.008-16	0.02-0.13	≤0.008-0.25
%WT/%NWT	92.6/7.4	96.3/3.7	100/0	96.3/3.7	100/0		100/0	
<b><i>A. clavatus</i> (1)</b>								
MIC	0.5	2	2	1	0.5	0.03	0.13	0.008

AMB: amphotericin B; VRC: voriconazole; POS: posaconazole; ITC: itraconazole; ISA: isavuconazole; ANF: anidulafungin; CSF: caspofungin; MCF: micafungin; GM: geometric mean; MIC: minimum inhibitory concentration ( $\mu\text{g/ml}$ ); MIC<sub>50</sub>: MIC causing inhibition of 50% of the strains tested; MIC<sub>90</sub>: MIC causing inhibition of 90% of the strains tested; WT: wild-type; NWT: non-wild-type.

(<http://www.ncbi.nlm.nih.gov/genbank/>), ISHAM ITS (<http://its.mycologylab.org/>), and MycoBank (<http://mycobank.org/>) online databases.

For cryptic species, multiple sequence alignment was performed using MUSCLE in MEGA v.7.0.26, and manual adjustments were made to improve it when needed.<sup>18</sup> This software was also used to conduct the Maximum likelihood analysis from the concatenated ITS, *BenA*, and *CaM* sequences of each strain. The evolutionary distances were computed using the Tamura-Nei method.<sup>19</sup> Support of the internal branches was assessed by the Bootstrap method with 1000 replications, where values equal to or higher than 70% were considered significant.

### Antifungal susceptibility testing

Broth microdilution testing was performed as outlined in the Clinical and Laboratory Standards Institute (CLSI) M38, 3rd ed. document.<sup>20</sup> The antifungal agents tested were: amphotericin B (AMB) (bioWORLD, USA), voriconazole (VRC) (Pfizer, New York, NY, USA), posaconazole (POS) (Merck, Rahway, NJ, USA), itraconazole (ITC) (Wako Pure Chemical Industries, Ltd., Japan), isavuconazole (ISA) (Cresemba®; Basilea, Bloomington, IN, USA), anidulafungin (ANF) (Equaltha®; Pfizer, Bedford, OH, USA), caspofungin (CSF) (Cancidas®; Merck, Whitehouse

Station, NJ, USA), and micafungin (MCF) (Mycamine®; Astellas Pharma, Deerfield, IL, USA).

Two-fold serial dilutions were prepared in dimethyl sulfoxide for each antifungal, further dilutions were made in RPMI-1640 with L-glutamine and buffered with 165 mM MOPS (Hardy Diagnostics, USA), reaching final concentrations that ranged from 0.03 to 16  $\mu\text{g/ml}$  for AMB and azoles, and from 0.008 to 4  $\mu\text{g/ml}$  for echinocandins. Fungal inocula were prepared from 7-day-old cultures grown on PDA and were adjusted spectrophotometrically. In brief, 100- $\mu\text{l}$  of each inoculum was diluted in RPMI-1640 and inoculated in U-bottom wells of 96-well plates (Costar, Corning Incorporated, NY, USA) containing 100  $\mu\text{l}$  of the drug dilutions. The final inoculum concentration ranged from  $0.4 \times 10^4$  to  $5 \times 10^4$  colony-forming units (CFU)/ml.<sup>20</sup> Finally, inoculated plates were incubated at 30°C and visually read after 24 and 48 h.

The minimum inhibitory concentration (MIC), defined as the lowest drug concentration that resulted in 100% growth inhibition, was determined visually with an inverted mirror after 48 h of incubation for AMB and azoles; whilst the minimum effective concentration (MEC), defined as the lowest drug concentration that produces a visible change in the morphology of the hyphae compared to the growth control well, was determined after 24 h of incubation only for echinocandins using an inverted

microscope. *Paecilomyces variotii* ATCC MYA-3630 and *Candida krusei* ATCC 6258 were used as quality-control strains.

The MICs obtained for every antifungal, excepting anidulafungin and micafungin, were interpreted according to the epidemiological cut-off values (ECVs) already established by the CLSI.<sup>21</sup> MIC  $\leq$  ECV isolates were considered as wild-type (WT), while MIC  $>$  ECV decreased susceptibility was contemplated and isolates were classified as non-wild-type (NWT), defined as potentially harboring resistance mechanism. Due to the lack of ECVs for *Aspergillus* cryptic species, MICs were presented for each of these species.

## Statistics

Descriptive and comparative analyses were done. Differences in species distribution were determined with a Z test using R statistical software version 3.6.1 for Windows (Institute for Statistics and Mathematics, Vienna, Austria). *P*-values  $<$  0.05 were considered statistically significant.

## Results

### *Aspergillus* species distribution

During the study period, a total of 146 clinical and 54 soil isolates of *Aspergillus* were collected and screened to determine the frequency and distribution of species. Among the clinical isolates, nine sections were located by ITS sequencing and were distributed as follows: *Fumigati* (47 isolates, 32.2%), *Flavi* (37 isolates, 25.3%), *Nigri* (33 isolates, 22.6%), *Terrei* (19 isolates, 13%), *Versicolores* (5 isolates, 3.4%), *Usti* (2 isolates, 1.4%) and finally, *Nidulantes*, *Circumdati*, and *Clavati* with just one isolate (0.7% each). Complementary, *BenA*, and *CaM* sequencing resulted in the identification of 12 cryptic species among 37 clinical isolates (Supplementary Fig. 1). Thus, 25% of the studied clinical isolates were found to be cryptic species and most of these were recovered from sputum (Supplementary Table 1). Seventeen of these isolates belonged to the section *Nigri* (13 *A. tubingensis*, 3 *A. aculeatus*, and 1 *A. aculeatinus*), six to section *Flavi* (5 *A. tamaritii* and 1 *A. nomius*), five to section *Fumigati* (4 *A. fumisynnematus* and 1 *A. hiratsukae*), five to section *Versicolores* (4 *A. sydowii* and 1 *A. griseoaurantiacus*), two to section *Usti* (2 *A. calidoustus*), and one each to sections *Nidulantes* and *Circumdati* (*A. rugulosus* and *A. ochraceus*, respectively). A 10% of the IA cases were proven, 80% probable and 10% possible. *A. fumigatus* was the main agent of invasive pulmonary aspergillosis (Table 2). Only six clinical isolates (4.1%) were considered colonizations, a half of that due to *A. flavus* (Table 2).

Regarding the soil isolates, six *Aspergillus* sections were found; isolates belonging to the section *Nigri* were the most frequent (21 isolates, 38.9%), followed by *Flavi* (12 isolates, 22.2%), *Terrei* (9 isolates, 16.7%), *Fumigati* (8 isolates, 14.8%) and last, *Nidulantes* (3 isolates, 5.5%) and *Circumdati*

**Table 2.** Agents of aspergillosis by clinical entity.

Clinical entity	Causal agents (%)
Invasive pulmonary aspergillosis	<i>A. fumigatus</i> (32) <i>A. flavus</i> (18) <i>A. terreus</i> (13) <i>A. niger</i> (11) <i>A. tubingensis</i> (10) Others (15)
Invasive non-pulmonary aspergillosis	<i>A. fumigatus</i> (25) <i>A. flavus</i> (25) <i>A. niger</i> (25) <i>A. hiratsukae</i> (25)
Sinusitis	<i>A. flavus</i> (36) <i>A. fumigatus</i> (27) <i>A. niger</i> (18) Others (19)
Otomycosis	<i>A. flavus</i> (33) <i>A. terreus</i> (33) Others (34)
Onychomycosis	<i>A. terreus</i> (50) <i>A. sydowii</i> (50)
Colonization	<i>A. flavus</i> (50) Others (50)

(1 isolate, 1.9%). Nine cryptic species were identified among 23 soil isolates (Supplementary Fig. 1), representing 43% of the total analyzed soil samples. Ten of these isolates belonged to section *Nigri* (8 *A. tubingensis*, 1 *A. costaricaensis*, and 1 *A. luchuensis*), seven to section *Flavi* (6 *A. tamaritii* and 1 *A. parasiticus*), three to section *Nidulantes* (3 *A. quadrilineatus*), and one each to sections *Terrei*, *Fumigati*, and *Circumdati* (*A. carneus*, *A. lentulus*, and *A. roseoglobosus*, correspondingly) (Supplementary Fig. 1).

A significant difference was found between clinical and soil isolates for *A. fumigatus* ( $P = 0.02$ ), *A. flavus* ( $P = 0.022$ ), and *A. tamaritii* ( $P = 0.011$ ). While members of the section *Fumigati* were isolated more frequently from clinical patient specimens, members of section *Nigri* were mainly recovered from soil. Isolates from sections *Versicolores*, *Usti*, and *Clavati* were detected only in clinical isolates.

### Antifungal susceptibility patterns

The results of susceptibility testing are presented in Tables 1 and 3. In Table 1 only data for the 140 non-cryptic *Aspergillus* species are displayed, while antifungal susceptibilities of the 60 cryptic species are shown in Table 3. The frequency of NWT isolates for AMB was 32%, just like the cumulative frequency for the azoles tested. For CSF, 13 isolates (9%) were considered as NWT according to the ECV breakpoints. Sixty-six percent of the NWT isolates were recovered from clinical specimens. Multi-azole decreased susceptibility was detected only in seven isolates (5% cross-resistance): *A. terreus* MAHU-1940 (MIC = 4  $\mu$ g/ml for VRC and ITC), *A. terreus* MAHU-1948 (MIC = 4  $\mu$ g/ml

**Table 3.** Antifungal susceptibilities of 60 cryptic *Aspergillus* spp.

Section	Species	Strain ID	MIC ( $\mu\text{g/mL}$ )							
			AMB	VRC	POS	ITC	ISA	ANF	CSF	MCF
<i>Nigri</i>	<i>A. tubingensis</i>	18-1934	0.5	1	0.125	0.5	1	<0.008	0.031	0.016
	<i>A. tubingensis</i>	16-2059	1	0.5	0.125	0.5	0.5	0.016	0.125	0.016
	<i>A. tubingensis</i>	20-0001	2	1	0.25	0.5	4	0.063	0.063	0.063
	<i>A. tubingensis</i>	MAHU-1987	2	4	1	2	0.25	0.016	0.063	0.063
	<i>A. tubingensis</i>	MAHU-1977	2	0.5	1	2	1	0.031	0.063	0.063
	<i>A. tubingensis</i>	MAHU-1971	1	0.5	0.5	0.5	0.5	0.016	0.031	0.008
	<i>A. tubingensis</i>	19-2716	0.5	1	0.25	1	1	0.063	0.063	0.016
	<i>A. tubingensis</i>	14-2653	0.5	1	0.125	0.5	1	<0.008	0.063	<0.008
	<i>A. tubingensis</i>	19-2097	0.5	2	1	1	2	<0.008	0.063	0.125
	<i>A. tubingensis</i>	MAHU-1953	1	1	0.5	4	2	0.008	0.063	0.016
	<i>A. tubingensis</i>	19-2195	0.5	0.5	0.125	0.25	1	0.016	0.125	0.031
	<i>A. tubingensis</i>	MAHU-1906	2	2	0.25	4	0.25	0.008	0.063	0.063
	<i>A. tubingensis</i>	FM-1027	0.5	1	0.125	0.25	4	0.063	0.031	0.016
	<i>A. tubingensis</i>	FM-1030	0.5	1	0.125	0.25	4	0.016	0.031	0.016
	<i>A. tubingensis</i>	HGM-017B	0.5	0.5	0.25	0.25	2	0.250	0.125	<0.008
	<i>A. tubingensis</i>	MAHU-1963	1	0.5	0.5	1	1	0.063	0.063	<0.008
	<i>A. tubingensis</i>	HGM-018	0.5	0.5	0.5	1	0.5	0.016	0.031	0.016
	<i>A. tubingensis</i>	MAHU-1952	1	1	0.25	1	1	0.008	0.016	0.063
	<i>A. tubingensis</i>	MAHU-1912	1	0.5	0.25	2	1	0.031	0.031	0.031
	<i>A. tubingensis</i>	FM-1026	0.5	1	0.25	2	1	0.016	0.031	<0.008
	<i>A. costaricensis</i>	17-3125	1	1	0.5	1	1	<0.008	0.031	0.031
	<i>A. costaricensis</i>	MAHU-1910	2	1	0.5	1	2	0.008	0.125	0.016
	<i>A. luchuensis</i>	MAHU-1937	2	1	2	4	0.5	<0.008	0.063	0.063
	<i>A. aculeatinus</i>	19-3593	0.125	0.063	0.125	0.125	0.031	<0.008	0.008	<0.008
	<i>A. aculeatus</i>	19-1260	0.25	0.063	0.125	0.016	0.125	<0.008	0.063	0.016
	<i>A. aculeatus</i>	17-2778	0.5	0.016	0.5	1	0.031	0.016	0.008	0.008
<i>A. aculeatus</i>	19-1749	0.5	0.063	0.016	0.016	0.125	0.016	0.125	0.016	
<i>Terrei</i>	<i>A. carneus</i>	MAHU-1927	4	1	0.25	2	2	0.016	0.063	0.016
<i>Usti</i>	<i>A. calidoustus</i>	16-1931	4	4	2	2	1	0.25	0.5	0.125
	<i>A. calidoustus</i>	19-2190	1	1	0.125	0.25	0.5	0.008	0.125	0.008
<i>Nidulantes</i>	<i>A. quadrilineatus</i>	MAHU-1947	8	0.5	0.25	0.063	0.25	0.031	0.25	0.031
	<i>A. quadrilineatus</i>	MAHU-1942	4	0.063	0.125	1	0.125	0.031	0.125	<0.008
	<i>A. quadrilineatus</i>	MAHU-1928	4	2	0.5	0.25	0.5	0.031	0.25	0.031
	<i>A. rugulosus</i>	19-0866	2	1	0.125	0.25	0.5	0.008	0.125	0.016
<i>Versicolores</i>	<i>A. sydowii</i>	MAHU-1951	4	0.25	0.25	0.125	0.25	0.008	0.063	0.016
	<i>A. sydowii</i>	MAHU-1956	2	2	0.5	1	0.25	0.016	0.031	0.063
	<i>A. sydowii</i>	19-1341	2	0.25	0.25	0.25	0.25	0.063	0.250	0.125
	<i>A. sydowii</i>	19-2315	2	1	0.5	0.25	0.25	<0.008	0.063	<0.008
	<i>A. griseoaurantiacus</i>	HSJ-D2	8	0.5	0.25	0.063	0.25	0.031	0.25	0.031
<i>Flavi</i>	<i>A. nomius</i>	19-4461	1	1	0.125	0.125	2	0.031	0.063	0.063
	<i>A. parasiticus</i>	MAHU-1918	2	2	0.250	1	0.125	0.031	0.031	0.031
	<i>A. tamarii</i>	MAHU-1932	2	0.5	0.5	1	0.125	0.016	0.125	0.125
	<i>A. tamarii</i>	MAHU-1976	2	2	0.25	1	0.25	0.016	0.063	0.5
	<i>A. tamarii</i>	18-3869	2	0.5	1	0.125	0.125	0.031	0.063	0.25
	<i>A. tamarii</i>	17-0938	0.5	0.5	0.063	0.125	0.25	0.016	0.125	0.031
	<i>A. tamarii</i>	19-3237	1	1	0.125	0.125	0.25	0.016	0.063	<0.008
	<i>A. tamarii</i>	MAHU-1909	2	0.5	0.25	0.5	0.125	0.008	0.125	0.063
	<i>A. tamarii</i>	18-0027	0.5	0.5	0.016	0.5	0.25	0.016	0.25	0.008
	<i>A. tamarii</i>	MAHU-1936	2	2	0.125	0.5	1	0.016	0.063	0.25
	<i>A. tamarii</i>	19-4391	2	0.5	1	0.5	0.5	0.031	0.25	0.25
	<i>A. tamarii</i>	MAHU-1959	4	2	0.25	0.5	1	0.063	0.063	0.25
	<i>A. tamarii</i>	MAHU-1961	1	0.5	0.25	1	0.125	0.008	0.031	0.016

Table 3. Continued.

Section	Species	Strain ID	MIC ( $\mu\text{g}/\text{mL}$ )							
			AMB	VRC	POS	ITC	ISA	ANF	CSF	MCF
<i>Circumdati</i>	<i>A. roseoglobulosus</i>	MAHU-1949	4	0.5	0.5	0.125	0.25	0.063	0.5	0.063
	<i>A. ochraceus</i>	HGM-013	16	2	0.5	1	0.5	0.125	0.031	0.031
<i>Fumigati</i>	<i>A. hiratsukae</i>	19-2832	1	1	0.063	0.016	0.25	0.031	0.063	0.016
	<i>A. lentulus</i>	MAHU-1964	4	2	0.125	0.5	0.5	0.016	0.125	<0.008
	<i>A. fumisynnematus</i>	19-3084	4	0.25	1	0.5	0.5	0.016	1	0.250
	<i>A. fumisynnematus</i>	HGM-015	2	1	0.063	0.063	0.25	0.063	0.125	0.016
	<i>A. fumisynnematus</i>	19-3053	2	0.5	1	0.5	0.25	0.125	0.5	0.125
	<i>A. fumisynnematus</i>	19-3051	2	0.25	0.063	0.5	0.125	0.031	0.125	0.016

MIC: minimum inhibitory concentration; AMB: amphotericin B; VRC: voriconazole; POS: posaconazole; ITC: itraconazole; ISA: isavuconazole; ANF: anidulafungin; CSF: caspofungin; MCF: micafungin.

for VRC, 2  $\mu\text{g}/\text{ml}$  for ISA), *A. terreus* 19-0679 (MIC = 4  $\mu\text{g}/\text{ml}$  for VCZ, 2  $\mu\text{g}/\text{ml}$  for POS and ISA), *A. terreus* MAHU-1931 (MIC = 8  $\mu\text{g}/\text{ml}$  for VRC, 2  $\mu\text{g}/\text{ml}$  for POS, 4  $\mu\text{g}/\text{ml}$  for ITC), *A. terreus* MAHU-1954 (MIC = 16  $\mu\text{g}/\text{ml}$  for VRC, 2  $\mu\text{g}/\text{ml}$  for POS, >16  $\mu\text{g}/\text{ml}$  for ITC, 4  $\mu\text{g}/\text{ml}$  for ISA), *A. flavus* MAHU-1973 (MIC = 8  $\mu\text{g}/\text{ml}$  for VRC, 2  $\mu\text{g}/\text{ml}$  for POS, ITC and ISA), and *A. fumigatus* 18-2367 (MIC = 8  $\mu\text{g}/\text{ml}$  for VRC, 1  $\mu\text{g}/\text{ml}$  for POS, 2  $\mu\text{g}/\text{ml}$  for ITC, 4  $\mu\text{g}/\text{ml}$  for ISA).

Concerning cryptic species, AMB MICs of 4, 8, and 16  $\mu\text{g}/\text{ml}$  in 1 *A. fumisynnematus*/2 *A. sydowii*, 1 *A. quadrilineatus* and 1 *A. ochraceus*, respectively, are notable. Regarding azoles, elevated MICs were recorded for VRC (4  $\mu\text{g}/\text{ml}$ ), POS (1  $\mu\text{g}/\text{ml}$ ), and ISA (2  $\mu\text{g}/\text{ml}$ ) in 1 *A. calidoustus*, 2 *A. tamaritii*/2 *A. fumisynnematus*, and 1 *A. carneus*/1 *A. nomius*, correspondingly. High MICs were found for *A. fumisynnematus* 19-3084 to AMB (4  $\mu\text{g}/\text{ml}$ ), POS (1  $\mu\text{g}/\text{ml}$ ) and CSF (1  $\mu\text{g}/\text{ml}$ ). One isolate of *A. lentulus* (MAHU-1964) with decreased susceptibility to AMB (4  $\mu\text{g}/\text{ml}$ ) and VRC (2  $\mu\text{g}/\text{ml}$ ) was detected.

## Discussion

This epidemiological study presents a general overview of the species distribution and antifungal susceptibility patterns of *Aspergillus* spp. from clinical specimens and soil samples in Mexico. The essential first step for the detection and monitoring of antifungal resistance in *Aspergillus* spp. is precise species identification, as this helps to identify intrinsically resistant species. Certainly, species identification to the complex level for clinically relevant isolates from patients requiring antifungal treatment is strongly recommended by the ESCMID-ECMM-ERS *Aspergillus* guideline.<sup>22</sup> In the present study, *A. fumigatus* (24.5%) was the predominant species, followed by *A. flavus* (18%), and *A. terreus* and *A. niger* (13.5% each). We found that the prevalence of *A. fumigatus* was lower than the 48.5% reported in the FILPOP study<sup>23</sup> and the 57.5% communicated in the FUNGAE-IFI study.<sup>24</sup> In other surveys, *A. flavus* was the second species in number of isolates (12.6–22.6%),<sup>4,25,26</sup> agreeing closely with

our data. The rate of *A. terreus* in our collection is similar to the FUNGAE-IFI study (13.8%),<sup>24</sup> and these data are higher than Brazil<sup>4</sup> and transplant centers in the United States.<sup>25</sup> On the other hand, we found that the prevalence of *A. niger* is comparable to that recently communicated in multicenter studies in Spain,<sup>23,24</sup> disagreeing with other geographic regions.<sup>4,25</sup>

Overall, cryptic species of *Aspergillus* represented 30% of our collection, which is considerably high compared with other reports.<sup>4,23–25</sup> *A. tubingensis* and *A. tamaritii* were the two cryptic species most commonly found within the sections *Nigri* and *Flavi*, respectively. These findings are in accordance with a survey in Spain<sup>24</sup> and Brazil.<sup>4</sup> In contrast to Castro et al.,<sup>24</sup> the number of *A. tubingensis* isolates was lower than *A. niger*, a concordant finding also communicated by Alastruey-Izquierdo et al.<sup>23</sup> Whereas in section *Fumigati*, three cryptic species were identified: *A. fumisynnematus*, *A. hiratsukae*, and *A. lentulus*; in section *Terrei*, *A. carneus* was the only one detected. These rare species and many others belonging to the sections *Versicolores*, *Nidulantes*, *Usti*, and *Circumdati* detected in this study have been already described in the literature as agents of human infections.<sup>7,27–30</sup>

In terms of antifungal susceptibility, we found 32% of NWT isolates to AMB according to the ECVs, which is elevated in comparison with the 3.4 and 10.8% reported by the FUNGAE-IFI study<sup>24</sup> and the FILPOP study,<sup>23</sup> respectively. MIC values higher than ECVs were more frequent in *A. fumigatus* (61.2%) and *A. terreus* (33.3%) and less common in *A. flavus* (8.3%) and *A. niger* (7.4%), agreeing with other authors that reported 27% of *A. terreus* and 14.8% of *A. flavus*.<sup>23,31</sup> Although decreased susceptibility to AMB has been regularly observed in *A. terreus* and to some extent in *A. flavus*,<sup>32,33</sup> AMB resistance has rarely been reported in clinical and environmental isolates of *A. fumigatus*. A large-scale study involving eight medical centers in Canada, Spain, the United States, and the United Kingdom communicated in 2011 a frequency of 5.6% (225/3,988) of *A. fumigatus* clinical isolates resistant to AMB.<sup>34</sup> Later, Reichert-Lima et al.<sup>35</sup> reported in 2018 that 27% (46/168) of the Brazilian

*A. fumigatus* isolates they studied exhibited MICs > 2 µg/ml. Despite the 61.2% of NWT *A. fumigatus* isolates we found, this is considerably high in comparison with the aforementioned reports. This percentage is well below the 96.4% recently communicated by Ashu et al.<sup>36</sup> in Hamilton, Canada. Furthermore, a significant difference in AMB susceptibility between clinical and soil NWT *A. fumigatus* isolates was detected, with the clinical isolates being more resistant to AMB (six isolates had MIC ≥ 8 µg/ml) than those recovered from soil; this agrees with Ashu et al.<sup>36</sup> and differs from Araujo et al.<sup>37</sup> Notwithstanding, the mechanism of AMB resistance in *A. fumigatus* remains unknown at present. These findings have important implications for the empiric use of AMB in the treatment of invasive aspergillosis by this particular species. On the other hand, even though intrinsic or possible resistance to AMB has been described in certain *Aspergillus* cryptic species<sup>23,31</sup> when compared to the present results, only one strain of *A. fumisynnematus*, one strain of *A. lentulus*, two strains of *A. sydowii*, one strain of *A. quadrilineatus*, and one strain of *A. ochraceus* were recorded.

Azole resistance among *Aspergillus* spp. has increased over the years.<sup>9,23,26</sup> Particularly in Mexico, Gonzalez-Lara et al.<sup>12</sup> communicated in 2019 the first report of azole-resistant *A. fumigatus* in 2 of 24 clinical isolates they screened. In this study, MICs of VRC, POS, ITC, and ISA were determined with an overall rate of NWT isolates of 32%, which is higher than that reported by the FUNGAE-IFI study,<sup>24</sup> but in accordance with those percentages previously communicated by other authors (10–55%).<sup>9,23,38</sup> VRC was the azole with the highest number of NWT isolates detected. Elevated VRC MICs for *A. fumigatus* have been previously observed<sup>39,40</sup> and we found six isolates with MICs ≥ 8 µg/ml (3 *A. terreus*, 1 *A. flavus*, and 2 *A. fumigatus*). Concerning POS, high MICs were detected in 8.6% of cases, in contrast to the 1.4% reported by Castro et al.<sup>24</sup> and agree with the 12.7% found by Alastruey-Izquierdo et al.<sup>23</sup> Moreover, nine isolates exhibited MICs above the ECV for ITC, 44% of which corresponded to *A. fumigatus*, as previously shown in other studies.<sup>41,42</sup> Regarding ISA, 6.1% of *A. fumigatus* isolates were considered as NWT, lower than the 11.3% reported by Messer et al.<sup>43</sup> in a screening they performed on 436 *Aspergillus* spp. isolates. On the other hand, cross-resistance to different azoles has been demonstrated<sup>9,24,39,42</sup> and we found five isolates of *A. terreus*, one *A. flavus*, and one more of *A. fumigatus* multi-azole-resistant.

Notably, 85% of all cryptic species were susceptible to the azoles tested. The exceptions were isolates representative of *A. carneus*, *A. tamarii*, *A. nomius*, *A. fumisynnematus*, *A. lentulus*, *A. tubingensis*, and *A. calidoustus* which were less susceptible to at least one triazole. Nevertheless, resistance to azoles has already been documented in cryptic species of *Aspergillus* section *Usti*,<sup>7</sup> section *Fumigati*,<sup>27,44</sup> and section *Nigri*.<sup>23,45</sup>

ANF, CSF, and MCF showed excellent activity against the *Aspergillus* isolates evaluated in this study. Of note, 1 *A. fumigatus*

and 12 *A. terreus* (8 clinical/5 soil isolates) exhibited MECs for CSF above the ECV. Our results are comparable to MEC values reported by other authors.<sup>46,47</sup> In addition, all cryptic species were susceptible to the echinocandins tested, except for 1 isolate of *A. fumisynnematus* which showed an MEC of 1 µg/ml for CSF, disagreeing with Alcazar-Fuoli et al.<sup>27</sup> Contrary to the increased MECs previously reported for *A. lentulus*,<sup>8</sup> the unique isolate of this species we detected was susceptible *in vitro* to the three echinocandins in accordance with Lockhart et al.<sup>47</sup>

This is an epidemiological study with the largest number of *Aspergillus* isolates analyzed in Mexico and represents an effort to describe the species distribution in both clinical and environmental settings (soil), as well as their antifungal susceptibility patterns. Assessment of local or regional epidemiology will help to adapt local therapeutic guidelines since experts have recommended considering moving away from triazole monotherapy when the resistance frequency exceeds 10%.<sup>48</sup> It highlights the importance of the implementation of a surveillance network to detect azole-resistant *Aspergillus* spp. at varying locations.

## Supplementary material

Supplementary data are available at [MMYCOL](https://www.mycologyonline.com) online.

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## Authorship contributions

R.J.T.-R., H.V.-L. and G.M.G. conceived and designed the study. H.V.-L., A.B., L.R.C.-O., M.F.M.-R. and J.A.-G. collected clinical isolates and data. A.A. and A.M.M. performed the molecular identification of isolates. R.J.T.-R. and H.V.-L. did the phylogenetic analysis. M.A.B.-G. and H.-V.-L. conducted the susceptibility testings. R.J.T.-R., A.B., L.R.C.-O., M.F.M.-R., J.A.-G. and G.M.G. analyzed data. R.J.T.-R. and H.V.-L. wrote the draft of the manuscript. All authors reviewed, revised, and approved the final version of the manuscript.

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## Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of this paper.

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