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Antifungal susceptibility of *Aspergillus* genus determined by the Etest® method: eleven years of experience at the Instituto Médico La Floresta. Caracas, Venezuela.

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Keywords: susceptibility; *Aspergillus* spp; cryptic species; antifungals; Etest diffusion method; minimal inhibitory concentration.

Abstract. This research aimed to determine the susceptibility of *Aspergillus* spp. to four antifungal agents using the Etest® method in several clinical samples (respiratory samples, soft tissue, otic tissue, and ocular tissue, among others) from a private health center in Venezuela. Thirty-three strains were evaluated: 11 *Aspergillus* section *Flavi*, eight *Aspergillus* section *Fumigati,* six *Aspergillus* section *Nigri*, four *Aspergillus* section *Terrei*, and four *Aspergillus* spp. A 0.5 McFarland standard suspension of a 5-day culture of each *Aspergillus* strain was prepared on Potato Dextrose agar and then inoculated on Sabouraud agar plates with 2% glucose. Voriconazole (VCZ), amphotericin B (AMB), caspofungin (CAS), and posaconazole (PCZ) were tested. Minimal inhibitory concentrations (MIC) in μg/mL were determined after 24 and 48 hours of incubation at 35 °C and th range (R), geometric mean (GM), $MIC_{50,}$ and MIC_{90} were calculated. The results for the 33 *Aspergillus* spp. tested after 24 h were the following: VCZ (R = 0.031- 16; GM = 0.145; MIC_{50} = 0.125 and MIC_{90} = 0.5), AMB (R = 0.031-16; GM = 0.644; MIC_{50} = 0.5 and MIC_{90} = 8), CAS (R = 0.031-16; GM = 0.1076; MIC_{50} = 0.063 and MIC_{90} = 1), PCZ (R =0.031 - 0.5; GM = 0.0755; MIC_{50} = 0.063 and MIC_{90} = 0.25). This investigation allowed assessing the antifungal susceptibility profiles of *Aspergillus* spp. isolated from clinical samples by the Etest® method, which is practical, reproducible and easy to perform in microbiology laboratories.

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Susceptibilidad a los antifúngicos del género *Aspergillus* determinada por el método Etest®: once años de experiencia en el Instituto Médico La Floresta. Caracas, Venezuela.

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Palabras clave: susceptibilidad; *Aspergillus* spp.; especies crípticas; antifúngicos; método de difusión Etest; concentración mínima inhibitoria.

Resumen. El objetivo de esta investigación fue determinar la susceptibilidad de *Aspergillus* spp., a cuatro antifúngicos mediante el método de Etest®, en aislados clínicos (muestras respiratorias, partes blandas, óticas, y oculares, entre otras) provenientes de un centro de salud privado en Venezuela. Se evaluaron 33 cepas: 11 *Aspergillus* sección *Flavi*, ocho *Aspergillus* sección *Fumigati*, seis *Aspergillus* sección *Nigri*, cuatro *Aspergillus* sección *Terrei* y cuatro *Aspergillus* spp. Se preparó una suspensión al 0,5 MacFarland a partir de cultivos de 5 días de incubación de cada cepa de *Aspergillus* en agar Papa Dextrosa, que se inocularon posteriormente en placas de agar Sabouraud con glucosa al 2%. Los antifúngicos ensayados fueron: voriconazol (VCZ), anfotericina B (AMB), caspofungina (CAS) y posaconazol (PCZ). Posterior a la incubación a 35 °C, se determinó la Concentración Mínima Inhibitoria en μg/mL (CMI) para cada antifúngico a las 24 y 48 h. Se calculó el rango (R), media geométrica (MG), CMI_{50} y CMI_{90}. Los resultados a las 24 h para las 33 cepas de *Aspergillus* fueron: VO (R = 0,031- 16; MG = 0,145; CMI_{50} = 0,125 y CMI_{90} = 0,5), AB (R = 0,031-16; MG = 0,644; MIC_{50} = 0,5 y MIC_{90} = 8), CS (R = 0,031-16; MG = 0,1076; MIC_{50} = 0,063 y MIC_{90} = 1), PO (R =0,031 - 0,5; MG = 0,0755; MIC_{50} = 0,063 y MIC_{90} = 0,25). Esta investigación permitió valorar los perfiles de susceptibilidad antifúngica en aislamientos clínicos de *Aspergillus* spp., mediante el método de Etest®, el cual es práctico, reproducible y fácil de realizar en los laboratorios de microbiología.

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INTRODUCTION

There has been a recent increase in epidemiological changes in filamentous fungi that cause diseases related to cryptic *Aspergillus* species. These species comprised 10 to 15% of *Aspergillus* isolates in epidemiological inquiries from Spain and the United States, particularly as the cause of invasive aspergillosis (IA) [1-3]. They are referred to as "cryptic" due to being sister species whose morphological distinction is rather complex, as they exhibit different phenotypic and genotypic characteristics[1].

Molecular studies have shown how the conventional identification method, based on morphological characteristics, is limited when it comes to differentiating *Aspergillus* species, as evidenced by the fact that such methodologies could only use one species or section (such as *Fumigati, Flavi, Nidulantes, Usti,* and *Terrei*) to identify morphologically identical species that could be separated through molecular methods [4].

The *Aspergillus* species most frequently isolated in a clinical context are *A. fumigatus*, *A. flavus*, *A. niger*, and *A. terreus*. The members of the *Fumigati* section, consisting of *A. fumigatus sensu stricto* and its cryptic species, are the most commonly isolated from clinical specimens and often from environmental sources. Furthermore, resistance to azoles has increased among clinical samples of the *Fumigati* section [5].

The prophylaxis and treatment of invasive aspergillosis are controversial due to its increasing morbidity and mortality [6]. While voriconazole (VCZ) is the drug of choice, isavuconazole (ISZ) can be used against *Aspergillus* spp., and is considered the most effective by European guidelines [7,8]. Posaconazole (PCZ) is recommended for primary antifungal prophylaxis during induction chemotherapy, immunosuppressive therapy for graft-versus-host disease after hematopoietic stem cell transplantation (HSCT), and salvage therapy for refractory IA [1–5]. Lipid formulations of amphotericin B (AMB) and echinocandins are an alternative to azoles in aspergillosis treatment [9]. However, epidemiological changes, including cryptic *Aspergillus* species' resistance to azoles, are of growing concern [4].

This study evaluated the levels of azoles (VCZ, PCZ), echinocandins (CAS), and amphotericin B susceptibility in *Aspergillus* species found in human samples using the Etest® gradient diffusion method.

MATERIAL AND METHODS

Aspergillus isolates

Clinical isolates of *Aspergillus* spp. were collected during 11 years (2011-2021) from patient samples processed in the Instituto Médico La Floresta microbiology laboratory in Caracas, Venezuela. Each clinical sample came from a different patient. The age, gender, and underlying disease of each patient were recorded. The isolates were preserved in distilled water with glycerol until the moment of the study. The different *Aspergillus* species' identification was based on the criteria by De Hoog *et al.* [10] and Klich *et al.* [11], assessing macro and microscopic aspects from subcultures on Sabouraud Dextrose Agar (SDA-Oxoid, USA), Mycosel Agar (Oxoid, USA), and Potato Dextrose Agar (PDA-Oxoid, USA), incubated in a temperature range between 20-30 °C.

In vitro susceptibility using the gradient diffusion method Etest®

A subculture on PDA agar of each *Aspergillus* spp. isolates were made and incubated for five days to prepare a conidia suspension in 0.85% sterile saline solution. The conidia concentration was determined by a Neubauer counting chamber (Hausser Scientific, Horsham, PA, USA) and standardized at 1 – 5 x 10^6 CFU/mL (Densimat™ bioMérieux, France) at 530 nm [12-14]. Plates containing Müeller-Hinton Agar, 2% glucose with Methylene blue, were inoculated, streaked in three directions, and left to dry for 15 minutes. Etest® strips of each antifungal (AB bioMérieux, France); VCZ, PCZ (0.002-32 μg/ mL), AMB, and caspofungin (CAS=0.016-256 μg/ mL) were placed according to the manufacturer's instructions. Each plate was incubated at 35 °C. MIC was measured at 24 h, with a maximum time of 48 h, in case the lecture was not possible at the stipulated time.

Criteria for interpreting the minimum inhibitory concentration

The MIC was defined as the lowest drug concentration at which the border of the elliptical inhibition zone intercepted the scale on the antifungal strip. To compare the MICs obtained during this study with the epidemiological cut-off values (ECVs) established by the Clinical and Laboratory Standards Institute (CLSI, M61 document, 2017), they were placed between two sequential dilutions taken to the subsequent higher dilution from the reference method. The values on the strip's upper end were taken to the highest concentration allowed, while those on the lower end were left unchanged. Ac-

cording to de CLSI, ECVs in wild and non-wild isolates are classified based on the following MICs: VCZ: *A. fumigatus*=1 μg/mL; *A. flavus, A. niger*, and *A. terrreus*=2 μg/mL. PCZ: *A. flavus*=0.5 μg/mL; *A. niger*=2 μg/mL, *A. terreus*=1 μg/mL. AMB: *A, flavus* and *A. terreus*=4 μg/mL; *A. fumigatus*, *A. niger*, and *A. versicolor*=2 μg/mL. CAS: *A. flavus* and *A. fumigatus*=0.5 μg/mL; *A. niger*=0.25 μg/mL, and *A. terreus*=0.12 μg/mL[15].

Statistical analysis

A database was created in Excel® 2010. The data was analyzed through percentages and central tendency measures: ranges, geometric mean (GM), mode (Mo), and median (Mdn) for each antifungal. The MIC values that inhibited 50% (MIC_{50}) and 90% (MIC_{90}) of the isolates were also calculated.

Quality control

American Type Culture Collection (ATCC®) control strains were used in order to evaluate the susceptibility tests: *A. fumigatus* ATCC® 204305, *Candida krusei* ATCC® 6258, and *Candida parapsilosis* ATCC® 22019.

RESULTS

The strains analyzed came from 33 patients, 18 female and 15 male, aged between 2-76 years and an average of 56 years. Thirty-three *Aspergillus* spp. isolates were identified, mostly from lower respiratory tract samples (17;51.5%), followed by isolates obtained from soft tissue (6;18.2%), ear discharge (4;12.12%), corneal ulcer scraping (2;6.06%), and one of each one from nasal septum, peritoneal fluid, bone marrow, and nail (1;3.03%). Table 1 shows *Aspergillus* species identified through phenotypic tests, isolation place, underlying disease, and MICs for each tested antifungal.

According to the ECV of CLSI, the results showed that 97% of *Aspergillus* isolates tested against VCZ were categorized as wild strains, while for PCZ, all the isolates were categorized as 100% wild strains. However, for AMB, 18.2% of isolates were wild strains.

Fig. 1 (A, B, C, D) shows the graphical distribution of each *Aspergillus* spp. against antifungals with their respective MICs. Table 2 describes the *in vitro* activity according to MICs, CMI_{50} and CIM_{90}.

DISCUSSION

Although it was found that the resistance of *Aspergillus* spp. tested in this study was low, without involving *Aspergillus* species with intrinsic resistance to some antifungals; it is necessary to be cautious when discussing susceptibility patterns in these species of filamentous fungi. The aim is to highlight the importance of monitoring resistance at local, national and international levels while investigating emerging resistance mechanisms [6].

Aspergillus flavus was the most frequent *Aspergillus* species isolated in this study, followed by *A. fumigatus*. This result is not comparable to that reported in the international literature, according to which *A. fumigatus* is the most identified species [1,4,14,16,17]. Susceptibility tests showed that 94% of *Aspergillus* species tested against VCZ had MICs lower than 1 μg/mL compared to the ECVs reported by CLSI, where these species were categorized as wild strains. However, one of the isolates MIC showed ≥16 μg/mL, which could be attributed to the fact that the *Fumigati* section contains *A. lentulus*, which has been observed to be intrinsically resistant to VCZ [18]. The molecular techniques corroborating this description were not feasible for this study. These results were similar to those reported by Castanheira *et al.*[17], who also obtained $MICs_{90}$ of 0.5 μg/mL in *A. fumigatus*, *A. terreus*, and *A. niger* against VCZ, as well as to those obtained by Espinell-Ingroff *et al.* [12]. As is the case for most azoles, VCZ acts on 14-α-sterol demethylase, and on 24-methylene dihydrolanosterol demethylase, another enzyme from the ergosterol biosynthetic pathway.

Table 1
Epidemiological, clinical characteristics and *in vitro* susceptibility to antifungal agents tested in *Aspergillus* spp. Isolates.

Nº	Type of sample	*Aspergillus*	Age	Gender	Diagnosis	VCZ (ug/mL)	AMB (ug/mL)	CAS (ug/mL)	PCZ (ug/mL)
1	Sputum	*A. fumigatus*	68	M	Lung cancer	0.064	0.5	0.125	0.031
2	Sputum	*A. niger*	62	F	Bile duct cancer	0.031	0.063	0.015	0.031
3	Sputum	*A. terreus*	62	M	Bile duct cancer	0.031	8	0.015	0.015
4	Nasal septum	*A. versicolor*	59	M	Lung cancer	0.5	0.5	1	0.250
5	Sputum	*A. terreus*	63	M	Lung cancer	0.25	4	0.125	0.500
6	Sputum	*A. fumigatus*	59	F	COPD	0.125	0.5	0.250	0.064
7	Sputum	*A. fumigatus*	70	F	Pneumonía	0.060	0.125	0.015	0.064
8	Ear discharge canal	*A. flavus*	45	F	Otitis media	0.064	0.5	0.064	0.125
9	Sputum	*A. fumigatus*	66	F	COPD	0.250	0.064	0.015	0.064
10	Foot discharge	*A. terreus*	69	F	Breast cancer	0.125	8	0.031	0.031
11	Bronchoalveolar lavage	*A. fumigatus*	57	F	Aspergilloma	≥16	≥16	0.064	0.5
12	Ear discharge canal	*A. flavus*	2	M	Otitis media	0.125	1	0.015	0.064
13	Jaw discharge	*A. fumigatus*	76	F	Reconstructive surgery	0.250	0.5	0.031	0.250
14	Ear discharge canal	*A. niger*	68	M	Otitis media	0.064	≥16	0.064	0.031
15	Thigh discharge	*A. penicellioides*	52	M	Trauma	0.250	2	0.031	0.063
16	Sputum	*A. flavus*	68	F	COPD	0.250	2	≥16	0.250
17	Sputum	*A. nidulans*	68	F	COPD	0.064	0.125	≥16	0.031
18	Sputum	*A. niger*	68	F	Breast cancer	0.031	1	0.125	0.031
19	Corneal ulcer	*A. flavus*	39	M	Keratitis	0.125	2	0.015	0.063
20	Ear discharge canal	*A. niger*	37	M	Otitis media	0.125	1	0.063	0.031
21	Peritoneal fluid	*A. penicellioides*	49	F	Renal insufficiency	0.250	1	0.5	0.031
22	Bronchoalveolar lavage	*A. flavus*	58	M	COPD	0.25	1	0.015	0.031
23	Sputum	*A. flavus*	57	F	Colon cancer	0.015	0.250	0.015	0.063
24	Endotracheal discharge	*A. flavus*	63	M	Lung cancer	0.015	0.125	0.031	0.063
25	Bronchial discharge	*A. flavus*	53	M	Pneumonia	0.063	0.5	0.015	0.125
26	Bone marrow	*A. fumigatus*	58	F	Lymphoid leukemia	0.5	0.5	0.015	0.125

Table 1
CONTINUATION

Nº	Type of sample	*Aspergillus*	Age	Gender	Diagnosis	VCZ (ug/mL)	AMB (ug/mL)	CAS (ug/mL)	PCZ (ug/mL)
27	Finger discharge	*A. niger*	61	F	Diabetes	0.063	0.125	0.015	0.031
28	Ankle tissue	*A. terreus*	42	F	Trauma	0.250	16	0.250	0.063
29	Nail	*A. flavus*	72	F	Diabetes	0.5	1	1	0.250
30	Sputum	*A. flavus*	68	M	Lung cancer/ COVID	0.5	1	0.063	0.250
31	Leg ulcer	*A. flavus*	81	F	Colon cancer	0.250	0.015	1	0.015
32	Sputum	*A. fumigatus*	18	M	Idiopathic hepatitis	1	0.5	0.25	0.063
33	Corneal ulcer	*A. niger*	35	M	Keratitis	0.031	2	0.063	0.250

VCZ: voriconazole; AMB: amphotericin B; CAS: caspofungin; PCZ: posaconazole, COPD: chronic obstructive pulmonary disease.

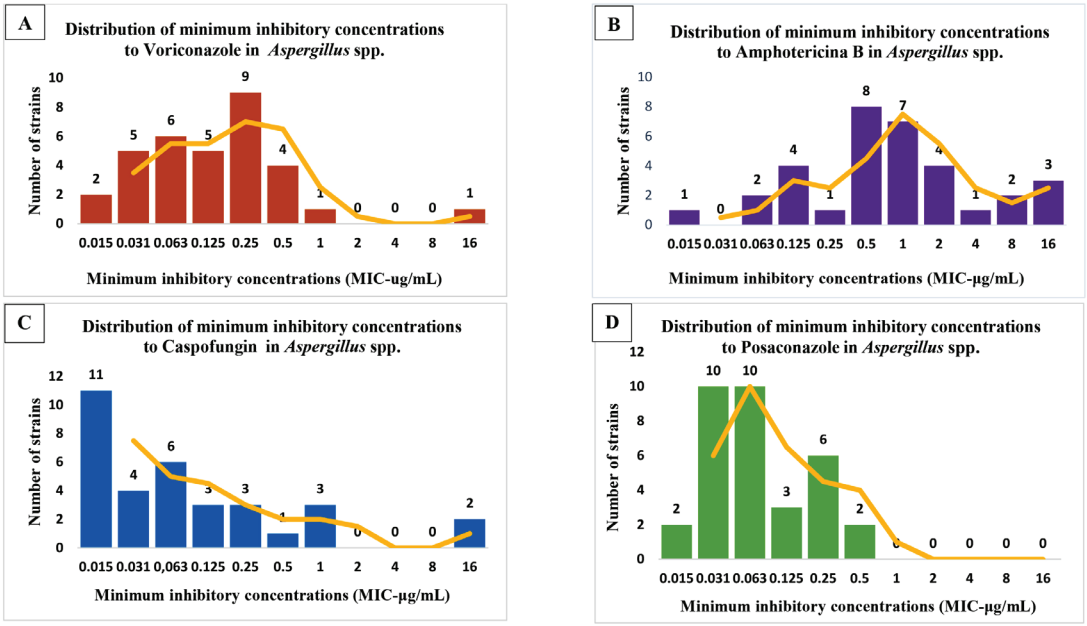

Fig. 1. Distribution of the different minimum inhibitory concentrations (MIC) obtained for *Aspergillus* spp. isolates (n=33), compared by antifungal agents tested. A) voriconazole; B) amphotericin B; C) caspofungin; and D) posaconazole.

This mechanism of action could explain the effectiveness of this antifungal compared to other azoles [19]. These drugs, VCZ in particular, are the first line of prophylaxis and treatment for fungal infections, although fluconazole is inactive against filamentous fungi [20].

PCZ is one of the last triazoles effective against various filamentous fungi, even Mucorales. Therefore, it has become the antifungal of choice in primary and salvage prophylaxis, especially for oncohematology patients [21]. The mean of the MIC (0.063 µg/mL), the MIC_{50} (0.063 µg/mL), and the

Table 2
Activity of antifungal agents tested by the E-Test® gradient diffusion method against *Aspergillus* spp. (n=33)

Antifúngicos	Range	Mean	Mode	Median	MIC_{50}	MIC_{90}
Voriconazole	0.015-16	0.015	0.25	0.125	0.125	0.5
Anphotericin B	0.015-16	0.1732	0.5	1	0.5	8
Caspofungin	0.015-16	0.0848	0.015	0.063	0.063	1
Posaconazole	0.015-0.5	0.0723	0.031	0.063	0.063	0.25

MIC_{50}: minimal inhibitory concentration that inhibited the growth of 50% of the isolates; MIC_{90}: minimal inhibitory concentration that inhibited the growth of 90% of the isolates. MIC: μg/mL.

MIC_{90} (0.25 μg/mL) obtained through this research shows the excellent *in vitro* activity of this triazole when compared to the ECVs reported by CLSI. The most frequently obtained MICs were 0.031 μg/mL and 0.063 μg/mL, although MICs for PCZ were relatively low. These results are similar to those obtained by Build *et al.* [22], confirming this drug's effectiveness in the tested isolates. However, that study suggests that high doses of PCZ could be used to treat azole-resistant *Aspergillus* spp. isolates.

Several studies have reported about the resistance of *A. fumigatus* to azoles. This is probably due to cross-resistance between triazoles used in agriculture [14,17,23,24]. These resistances are transmitted to humans through food and water consumption [9]. Most of them are mediated by the *cyp51A* gene. Depending on the specific mutation, one or even all triazoles can be resistant [4]. Resistance rates vary widely among medical centers worldwide, reporting high rates or rates of 1% or less [23-25]. MICs varied between Aspergillus species against AMB. Fortunately, resistance to this antifungal is very rare. Even so, the MIC was above the ECV reported by the CLSI in six of the Aspergillus species isolates. Four *A. terreus* isolates showed MICs ≥ 4 μg/mL, while MICs of both one *A. niger* isolate and one *A. fumigatus* were ≥ 2 μg/mL

The *Fumigati* section susceptibility profile is not consistent because this section contains *A. lentulus* and A. *fumigatiaffinis*, which have high MICs for azoles and AMB [26]. Despite this, it should be noted that data obtained from the *Fumigati* section regarding MICs were two dilutions lower than those reported by Denardi *et al.*[9] (Brazil) and Castanheira *et al.* [17] (global study).

Aspergillus terreus is known to be intrinsically resistant to AMB, but this depends on the cryptic species within the *Terrei* section [16]. Despite testing a few isolates, this study's A. terreus MICs results are comparable to those reported in the literature. *Aspergillus terreus* has emerged as an opportunistic pathogen, capable of causing pulmonary aspergillosis, onychomycosis, and fungal keratitis, among other diseases; it has also garnered attention due to its natural *in vitro* and *in vivo* resistance [19].

Amphotericin B is the antifungal of choice to treat severe fungal infections. Most hospitals or healthcare services commonly use it. The selective pressure in these environments could contribute to the emergence of resistant phenotypes. Resistance to AMB is most likely associated with low levels of ergosterol in the cell membrane, which reduces the effectiveness of the drug because of mutations in the *Erg3* gene that inactivate 5,6 sterol desaturase, an enzyme that functions as a step in the sterol biosynthetic pathway, creating dysfunctional sterols. There are also Aspergillus species capable of producing enzymes with reducing activity, decreasing the oxidative stress of AMB in fungal metabolism [28,29].

In this study, other isolates, such as A. niger and A. nidulans, were categorized as non-wild-type or AMB-resistant strains. In any case, although other studies have reported similar results, the number of isolates tested from these species was not significant enough to obtain sufficient data to draw more informed conclusions [7-29].

Echinocandins are one of the new antifungals used for aspergillosis treatment. These molecules inhibit the synthesis of β-(1,3)-d-glucan synthase, indirectly affecting β-(1,3)-d-glucan incorporation into fungal cell walls. Caspofungin is used successfully in salvage therapy against IA. During this study, 94% of *Aspergillus* species were resistant against CAS, and showed mean, MIC_{50}, and MIC_{90} values of 0.063 μg/mL, 0.063 μg/mL, and 1 μg/mL, respectively, when compared to the ECVs reported by CLSI. The GM of the *Aspergillus* spp. against CAS (0.063 μg/mL) is a lower dilution than that of Denardi *et al*. [9] (0.078 μg/mL) regardless of the methodology used. In the treatment of aspergillosis, echinocandins are focused mainly on the wall of the apical region of the *Aspergillus* hyphae, ignoring the rest of the fungal structures. The activity of this group of antifungals thus affects the growth rate of the fungus but leaves other physiological aspects intact [30]. Two other isolates, *A. flavus* and *A. nidulans*, showed MIC ≥ 16 μg/mL, categorizing them as non-wild. Resistance to echinocandins is not common among *Aspergillus* species; however, some recent reports of resistance to CAS [30,31] are consistent with our findings.

These cryptic species are significant mainly because they can display intrinsic resistance with an *in vitro* rate of around 40% against at least one antifungal [6, 13]. The resistance rate against azoles, polyenes, and echinocandins varies by region, hence the importance of getting global epidemiological data. Furthermore, MICs from environmental and clinical samples of azole-resistant *Aspergillus* species should be compared to understand this antifungal resistance phenomenon. In order to determine a precise ECV that could improve the use of clinical cut-off points for *Aspergillus* species, it seems imperative to obtain both more epidemiological and more semiotic data (clinical and molecular), which includes the treatment of IA caused by resistant strains to different antifungal drugs [1,17,23].

We ratify the need to identify the different species in each section using molecular techniques and include susceptibility tests. In this study, the Etest® agar strip diffusion method proved to help obtain ECV-guided MICs established by CLSI. These MICs provided clinical guidelines for treating infections caused by *Aspergillus* species isolated in Venezuela.

Conflict of interest

The authors declare they have no conflicts of interest.

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Authors Contribution

XM study conceptualization and design; research; analysis and interpretation of results; preparation, writing, review and editing of the final manuscript. CM and DO research; analysis, interpretation of results and final manuscript editing.

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