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Susceptibilidad antifúngica *in vitro* de aislamientos clínicos de *Fusarium* de Colombia

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ABSTRACT

Objective The aim of the present study was to evaluate the antifungal susceptibilities of isolates of *Fusarium* to amphotericin B, itraconazole and voriconazole.
Methods The susceptibility of 44 isolates of *Fusarium* was tested by the E-test methodology.
Results All the isolates were resistant to itraconazole, and 89 % and 54,5 % were resistant to amphotericin B and voriconazole, respectively.
Discussion The results confirm the high level of resistance reported, regardless of the species or the strain of *Fusarium* involved. The high MICs level observed are worrying and suggest that new drugs are needed.

Key Words: *Fusarium*; amphotericin B; voriconazole *(source: MeSH, NLM).*

RESUMEN

Objetivo Evaluar la susceptibilidad antifúngica *in vitro* de aislamientos de *Fusarium* a los antimicóticos amfotericina B, itraconazol y voriconazol.
Métodos La susceptibilidad de 44 aislamientos clínicos de *Fusarium* fue evaluada por el método de difusión en disco, E-test.
Resultados Todos los aislamientos fueron resistentes al itraconazol, y 89 % y 54,5 % fueron resistentes a la amfotericina B y al voriconazol, respectivamente.
Discusión Los resultados confirman el alto nivel de resistencia reportado, independiente de la especie o la cepa de *Fusarium* involucrada. Los valores tan altos de MICs son preocupantes y sugieren la necesidad de evaluar nuevos medicamentos.

Palabras Clave: *Fusarium*; anfotericina B; voriconazol *(fuente: DeCS, BIREME).*

Fungi of the genus *Fusarium* are primarily plant pathogens and saprobes that cause a broad spectrum of infections in humans; including superficial, local, invasive, and disseminated infections, in immunologically deficient humans (1). After aspergillosis, disseminated fusariosis is the second most common cause of invasive infection by filamentous fungi in patients with hematologic malignancies or those undergoing transplants of hematopoietic progenitors; its high mortality rate and the lack of an optimal management protocol have raised increasing interest in this mycosis (2).

The most frequent species causing fusariosis are *F. solani*, *F. oxysporum*, and *F. verticillioides* (1,3). Although less frequent, several other species also cause human infections. Some of these species are *F. chlamydosporum*, *F. dimerum*, *F. incarnatum* and also the following species that are included into the *Gibberella fujikuroi* species complex: *F. napiforme*, *F. nygamai*, *F. proliferatum*, and *F. sacchari* (4).

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F. temperatum has been recently reported as an agent of keratitis (5). However, the relevance of one species could change depending on the geographic area and the kind of infection involved. In the North of Italy, *F. verticillioides* was the most frequent isolated species from deep-seated infections and, *F. solani* was the most frequent isolated species from superficial infections (1). In Brazil, strains of *F. solani* have represented the 88 % of a total of 41 isolates involved in *Fusarium* keratitis (3), and in Bogotá (Colombia), *F. solani*, *F. oxysporum* and *F. verticillioides* represented the 64,9; 32,8; and 2,3 % respectively, from a total of 137 patient with onychomycosis by *Fusarium* (6).

Fusarium is one of the most genetically heterogeneous fungi groups. Many species of this genus, that were identified —based on morphological characters— proved to be species complexes with little to no morphological differences, rather than single species (7). Many species, as *F. solani*, *F. oxysporum*, *F. verticillioides*, *F. chlamydosporum* and *F. dimerum* represent complexes of species (4).

The huge genetic diversity of *Fusarium*, somehow is reflected in the susceptibility patrons to antifungals. Controversial results of susceptibility to antifungal and a high level of resistance are reported. Some species are less sensible than others, or strains of the same species have different levels of susceptibility to the same product (1,7). The *F. solani* species complex is one of the group with the poorest response, *in vitro* and in vivo to different drugs, as well as one of the most heterogeneous genetically speaking (1,8,9). The *F. fujikuroi* species complex showed resistance patterns species-specific (10).

The triazoles represent the frontline drugs for the treatment of mould diseases; nevertheless, emerging moulds (including *Fusarium* spp.,) may be less susceptible or resistant to these antifungals (11). Polyenes and azole compounds are routinely applied chemotherapy to fungal keratitis (12). Amphotericin B and voriconazole are the preferred drugs of choice for treatment of deep and disseminated infections, although some *Fusarium* species are not susceptible to them (7). However; good results have also been found, with better activity of the amphotericin B than the voriconazole (1,3,13) or voriconazole with better activity than amphotericin B (1,14).

Therefore, taking into account that the data of antifungal susceptibility of *Fusarium* spp are conflicting and could depend on the species, strain, kind of fusariosis and the antifungal drug (13), we have studied the susceptibility of 44 clinical isolates of *Fusarium* to amphotericin B, itraconazole and voriconazole by the E-test methodology. The results showed that all the *Fusarium* isolates were resistance to the itraconazole and 89 % of them to amphotericin B, too. Voriconazole had a moderate activity; only 15, 9 % of the isolates were sensible. These suggest that others antifungals should be considered.

METHODOLOGY

Isolates

The isolates were recovered from patients at the Corporación para Investigaciones Biológicas (CIB) in Medellin (Colombia) since 2004 to 2006. A total of 44 *Fusarium* isolates, from toenails (n=35), hand nails (n=2), skin (n=4) and cornea (n=1) were evaluated. These were identified as *Fusarium* spp, by the direct exam in Chinese ink and KOH at 20 %, and by their macroscopic and microscopic morphological features after they were cultured in the media Sabouraud, potato dextrose agar (PDA) and Mycosel at 23 ºC for one to three weeks. The identity to the specie level of 35 isolates was determined by partial sequence of the transduction elongation factor gene (TEF1a), in another work (15). All the isolates were preserved in sterile water at room temperature in darkness.

Antifungal susceptibility

The *in vitro* activity of amphotericin b, itraconazole and voriconazole was evaluated against 44 isolates of *Fusarium*, by the disk diffusion test according to the methods provided in CLSI M38-A (16,17).

The isolates were sub-cultured on PDA plates and incubated at 25 ºC for seven days. Each colony was recovered with 10 mL of distilled water into a glass sterile tube; and after sedimented for 20 min., the upper part of each tube was collected in a new sterile tube. The suspensions were adjusted to a transmittance of 68–70 % at 530 nm, with distilled water, corresponding to an inoculum of 106 UFC/mL. A volume of 200 µl of each inoculum was added onto plates with 16 mL of RPMI medium supplemented with 1,5 % of agar, 2 % of glucose, at pH 7, and 0,165 M of buffer MOPS (Morpholine propane sulfonic acid, AES laboratory, Paris, France). The inoculum was allowed to dry for 15–30 minutes.

The E-test method was performed by following the instructions of the manufacturer (Etest®-AB Biomérieux). The antifungal agents were tested in concentrations than ranged from 256 to 0,016 µg/mL; two strips with the antifungal concentration, were placed in opposite direction on the inoculum. These were cultured at 28 ºC. The MICs that produced inhibition of growth were read after 48 hours, by visual examination; MICs were recorded as the lowest drug concentration where the border of the inhibition ellipse intersects with the scale on the plastic antifungal strip. Candida krusei ATCC 6 258 was included as a quality control strain (18).

RESULTS

The results showed that all the 44 isolates of *Fusarium* evaluated, except the control (C. krusei) were resistant to itraconazole; 39 of the isolates (representing the 89 %) were also resistant to amphotericin B; the others five were intermediate or sensible dose-dependent (two of *F. oxysporum* and two of *F. solani*, and the other isolate —63 946— was not identified) (Table 1).

The voriconazole was the only antifungal that showed moderate activity, with seven isolates (representing the 15,9 %) sensible to the product (with MICs of less than 1 µg/mL); five of them were identified as *F. oxysporum* (the two remaining were not identified); 13 isolates (29,6 %) were sensible dose-dependent; six of *F. oxysporum*; three of *F. solani* and one of *F. incarnatum* (56 665); the three remaining were not identified. The others 24 isolates (representing the 54,5 %) were resistant to voriconazole; 10 of *F. oxysporum*; 10 of *F. solani*, and 4 that were no identified. All the isolates resistant to voriconazole were also resistant to amphotericin B and itraconazole. All of them were taken from nails, except for the isolate 56 988 of *F. solani*, which was taken from the cornea (Table 1). It is important to highlight the number of isolates with MICs higher than 32 µg/m: 44 (all the isolates), 36 and four to itraconazole, amphotericin B and voriconazole, respectively.

Table 1. Antifungal susceptibilities of clinical isolates of *Fusarium* to amphotericin B, itraconazole and voriconazole by the E-test method

Strain	Origen	Gender	Voriconazole	Itraconazole	Amphotericin B
Control	24 hours	-	0,125	0,125	0,047
Control	48 hours	-	0,25	0,75	1
55349	Toenails	F	1,5	>32	>32
55444	Toenails	M	2	>32	2
55496	Toenails	F	0,75	>32	>32
55529	Toenails	M	4	>32	>32
55583	unknown	M	1,5	>32	>32
55787	Toenails	F	1,5	>32	>32
55861	Toenails	F	2	>32	>32
55945	Toenails	F	1	>32	>32
56054	Skin	M	2	>32	3
56212	Toenails	F	1,5	>32	>32
56240	Toenails	F	6	>32	16
56242	Toenails	M	4	>32	>32
56301	Toenails	F	8	>32	>32
56363	Toenails	F	0,75	>32	>32
56665	Skin	F	1	>32	>32
56780	Toenails	F	4	>32	>32
56891	Toenails	M	2	>32	0,19
56894	Toenails	M	4	>32	4
56988	Cornea	M	8	>32	16
57221	Toenails	F	1,5	>32	>32
57560	Toenails	F	1	>32	>32
57855	Toenails	F	>32	>32	>32
57949	Toenails	F	1	>32	>32
57952	Toenails	F	0,5	>32	>32
63051	Skin	M	1,5	>32	>32
63447	Toenails	F	1	>32	>32
63550	Toenails	F	>32	>32	>32
63635	Toenails	F	2	>32	>32
63648	Toenails	F	0,5	>32	>32
63649	Toenails	F	8	>32	>32
63666	Hand nails	F	3	>32	>32
63746	Toenails	M	0,5	>32	>32
63749	Hand nails	M	8	>32	>32
63768	Toenails	F	2	>32	>32
63783	Toenails	F	>32	>32	>32
63786	Toenails	F	>32	>32	>32
63857	Skin	F	0,75	>32	>32
63868	Toenails	F	2	>32	>32
63880	Toenails	F	0,25	>32	3
63901	Toenails	F	6	>32	>32
63917	Toenails	F	8	>32	>32
63946	Toenails	F	1,5	>32	2
64938	Toenails	M	1,5	>32	>32
64945	-	-	16	>32	>32

DISCUSSION

Fusarium spp., is a well-known opportunistic fungal agent that can cause important infections in immunocompromised patients. It is also one of the main mycotoxigenic fungi (19). *Fusarium* is the leading pathogen of fungal keratitis in most of the studies worldwide, particularly in tropical regions (3); its ability to form biofilm was suggested as a contributing factor in recent outbreaks (9). *Fusarium* spp. have frequently been isolated from patients with onychomycosis, mainly of the specie *F. oxysporum* (6).

The typical profile of the antifungal susceptibility of *Fusarium* spp. is the resistance to most antifungal agents. Due to the susceptibility pattern of *Fusarium* spp., the antifungal therapy options are limited (20). Additionally, information on epidemiology, antifungal susceptibilities and correlation with clinical outcomes is lacking, and such information is useful from a prognostic, diagnostic and therapeutic viewpoint (3). On the other hand, MICs break points are not available for mold testing, therefore the isolates has been grouped as susceptible (MIC or MEC,<1µg/mL), intermediate (MIC or MEC, 2 µg/mL) and resistant (MIC or MEC, >4 µg/mL), based on reported *in vitro* data obtained with large numbers of isolates (17,21). The levels for the antifungal that we evaluated were: <1, 1-2, >2 µg/mL for sensible, sensible dose-dependent and resistant, respectively.

In our work, the epidemiological data showed that, from the 44 clinical isolates, 84 % were involved in onychomycosis and 70 % were taken from females, which suggest that the generalized practice of manicure and pedicure in Colombia could be contributing to the dispersion of *Fusarium* spp. The most prevalent specie was *F. oxysporum*, with 60 % (21 of 35 isolates previously identified), followed by *F. solani* with 37 % and *F. incarnatum* with one isolate, in agreement with previous reports (6).

The data of susceptibility of *Fusarium* spp., to antifungal drugs are conflicting (13); different works have shown that the susceptibility is species-related, with *F. solani* having the highest MICs values (1,8,9), or strain-related as those biofilm producers, over all of *F. solani* (3). The susceptibility to the same antifungal is variable. It seems that itraconazole has a poor activity against *Fusarium* spp., as we found in our work. In some cases, amphotericin B has shown better activity than voriconazole (1,3,13), or in some others, voriconazole is better than amphotericin B (14), in agreement with our work.

A better efficacy of the amphotericin B than itraconazole against strains of different clades of *F. solani* has been reported (8). Similarly, the amphotericin B has been shown as the most active drug against *F. solani*, while voriconazole and posaconazole were active against other *Fusarium* species (1). Strains of *F. solani* that produce biofilms has lower susceptibility, mainly for amphotericin B, which seems to be related with a worse clinical outcomes for *F. solani* compared with other *Fusarium* species (3).

In a study made in the United States of America it was found that, from the isolates involved in keratitis, the species of *F. solani* were the most common, followed by *F. oxysporum* species; and more strains of *F. solani* formed biofilm than strains of *F. oxysporum*, and the ability to form biofilm varied by strain and clade type (9). None of the isolates of *F. solani* of our work was sensitive to voriconazole; instead, there was of *F. oxysporum*, although the isolates came from patient with onychomycosis mainly.

Voriconazole has been used to treat fungal infections in immunocompromised patients, including those caused by *Fusarium* spp (6). In our work, voriconazole was the best of the three antifungals evaluated against *Fusarium* spp., although only the 15, 9 % and 29 %, 6 % of the isolates was sensible and sensible dose-dependent, respectively to the product. As we have said, all the sensitive isolates belong to *F. oxysporum* and, from the 13 isolates (29,6 %) sensibles dose-dependent, six were of *F. oxysporum*, three of *F. solani* and one of *F. incarnatum* (the remaining three were not identified), which suggest that *F. solani* strains are less sensible. However, equal number of strains (ten) of *F. oxysporum* and *F. solani* were found resistant to voriconazole. Similarly, in another study made in Colombia with 137 patients with onychomycosis by *Fusarium* spp., the highest MICs values with voriconazole were of the isolates of *F. solani*, followed by *F. oxysporum* and *F. verticillioides*; 83.9 % and 66.7 % of the *F. solani* and *F. oxysporum* isolates were resistant to voriconazole, respectively (6).

Fusarium spp. show higher MICs value compared to other genus (6). In a study made in Colombia, the *in vitro* activity of fluconazole, itraconazole, voriconazole and terbinafine against fungi causing onychomycosis by species of Candida, *Fusarium*, *Fusicoccum dimidiatum*, *Scytalidium hyalinum* and dermatophytes showed that the *Fusarium* species had the highest MIC values, with all the antifungal agents, compared to the other fungal genera (22). In agreement with our work, they found the highest MICs values with itraconazole to *F. solani* and *F. oxysporum*, while voriconazole showed lower values but, contrary to our results, the isolates of *F. oxysporum* were less sensible than those of *F. solani* (between 2-16 and 2-8 µg/mL, respectively) (22). Also, they reported more species of *Fusarium*: six isolates of *F. oxysporum*, two of *F. solani*, one of *F. proliferatum*, one *F. dimidiatum*, and

one of *F. nygamai* (22); however, the differences between the methods for identification of isolates between these two works should be considered.

A few works have compared numerous antifungal products against *Fusarium* spp. The antifungal susceptibilities from a strain collection of 48 isolates of *Fusarium*, belonging to the less-common *Fusarium* species of clinical interest, *F. chlamydosporum*, *F. dimerum*, *F. incarnatum*, *F. napiforme*, *F. nygamai*, *F. proliferatum*, and *F. sacchari* was evaluated against 11 antifungal drugs (including amphotericin B, itraconazole and voriconazole) (13). Terbinafine was the most active drug against all the species tested with the exception of *F. incarnatum*, for which amphotericin B was the most active; amphotericin B was the second most active drug and, voriconazole although showed poor activity against all the tested strains. It was the third most active antifungal drug (13).

In Brazil, the *in vitro* susceptibility of isolates of *F. napiforme* responsible for a disseminated fusariosis were evaluated against amphotericin B, itraconazole, voriconazole, micafungin, 5-flucytosine, miconazole and fluconazole. The isolates were resistant to amphotericin B, with MIC ranging from 2 to 4 µg/mL; the azoles were the most active against all the tested isolates (14).

In summary, the *in vitro* and in vivo activity against *Fusarium* species is not predictable. The unsatisfactory susceptibility profiles *in vitro* can be attributed to several factors, including the species of *Fusarium*, the strain, and the kind of antifungal drug. In vivo other factors are affecting too, as the kind of fusariosis and the underlying disease of the patient. Therefore, the choice of the antifungal should be determined on a case-by-case basis, depending on the species and susceptibilities performed at an experienced center, whenever feasible to obtain (22).

As it has been said "…despite of the methodological advance for determining antifungal susceptibility for fungi, the interpretation of the results and determination of how best to use these results continue to cause considerable confusion" (21), seems to be the best interpretation of the susceptibility of *Fusarium*. Therefore, categorical conclusions are impossible, but for our local area in Colombia, itraconazole should not be used for the treatment of fusariosis; nor amphotericin b, since any of the isolates was sensible to it. Voriconazole could be used but a test is always required ∗

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