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Brief Communication

In vitro activities of antifungal agents alone and in combination against fluconazole-susceptible and -resistant strains of *Candida dubliniensis*

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In the present study we used two groups of *Candida dubliniensis* strains: one containing fluconazole-susceptible clinical isolates and another containing fluconazole-resistant laboratory derivative from the former to examine the changes on susceptibility accompanying the development of resistance to fluconazole. Our findings confirmed the ability of *C. dubliniensis* isolates to become resistant to fluconazole and indicated that this resistance was crossed with ketoconazole, itraconazole, ravuconazole and terbinafine. We also tested combinations of terbinafine, amphotericin B, itraconazole and voriconazole against both groups of isolates in a checkerboard assay. Surprisingly, most combinations evidenced indifferent interactions, and the best synergism appeared when terbinafine and itraconazole were combined against the fluconazole-resistant group.

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The increasing incidence of fungal infections without a satisfactory response to the current antifungal therapy and the slow development of new agents with novel mechanisms of action have produced significant interest on associations between antifungal agents.¹ Achievement of synergy is one of the major theoretical justifications for combination therapy, since it may enable practitioners to diminish drug dosages, expand the coverage in seriously ill patients with mixed infections and delay emergence of resistant mutants. On the other hand, cost of therapy may increase, the chance of drug reactions is greater, one drug may antagonize the effect of the

other, and the combination may accomplish no more than one effective drug.²

Since *Candida* spp. are the most common cause of fungal infections, it stands to reason that *Candida albicans* has been the *Candida* spp. most commonly evaluated in the majority of in vitro antifungal combination studies. While the combination of azoles with amphotericin B has provided controversial results,³ most of studies have found additive or synergistic effect when azoles were combined with terbinafine against *C. albicans*.⁴⁻⁶ Other *Candida* spp. have also been evaluated,⁵ but little is known about the effects of antifungal combinations against *Candida dubliniensis*.

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C. dubliniensis resembles *C. albicans* in many phenotypic aspects and assumes importance since it is often associated with mucocutaneous candidiasis especially in HIV-infected patients, showing a pathogenic character of this group.⁷ Although previous studies indicate that most strains of *C. dubliniensis* are susceptible to the same antifungal spectrum, clinical fluconazole-resistant isolates have been reported.⁸ In order to search for new alternative therapies, the aim of this study was to evaluate in vitro effects of the combination terbinafine and amphotericin B with azoles against fluconazole-sensitive (FS) and -resistant (FR) strains of *C. dubliniensis*.

We used two groups of *C. dubliniensis* strains: the first included clinical fluconazole-susceptible isolates recovered from AIDS-patients. The second group included fluconazole-resistant derivatives obtained from the first through an in vitro method of induction of fluconazole resistance described by Fekete-Forgács et al.⁹ as follows: a 10 mL culture of the FS strain was grown overnight in Sabouraud glucose broth (SDB). Cells were added to flasks containing 20 mL of SDB to achieve a final absorbance of 0.1 ($\lambda = 640$ nm). The culture was incubated at 30°C for 10h, and then fluconazole was added at a final concentration of 8 $\mu\text{g/mL}$ (higher than the MIC, Table 1). After 14h of further incubation the cells of the fluconazole-containing culture were subcultured three times consecutively into fresh SDB containing 8 $\mu\text{g/mL}$ fluconazole and in each case were incubated at 30°C with shaking for 24h. After the third incubation cells were added to flasks containing 20 mL of SDB containing fluconazole 8 $\mu\text{g/mL}$ to achieve a final absorbance of 0.1. After 10h incubation the fluconazole was added at a final concentration of 16 $\mu\text{g/mL}$, and after 14h of further incubation the cells of this culture were subcultured three times into fresh SDB containing fluconazole 16 $\mu\text{g/mL}$ and incubated in each case at 30°C with shaking for 24h. The concentration of fluconazole was always duplicated under the procedure; the final concentration was 64 $\mu\text{g/mL}$. Cells from this culture were plated, and single colony was designated isolate FR.

Voriconazole (Pfizer Inc. – New York, NY, USA), itraconazole (Janssen-Cilag Pharmaceutica – Belgium) amphotericin B and fluconazole (Sigma Chemical Co. – St. Louis, MO, USA) and terbinafine (Novartis) were obtained as standard powders and prepared according to Clinical and Laboratory Standards Institute guidelines.¹⁰

Antifungal combinations against the two groups of isolates were tested in duplicate using checkerboard method. After determination of the MIC of each drug alone, 10 dilutions were prepared in order to obtain four-fold the final concentrations: 50 mL- aliquots of each azole dilution were combined with other 50 mL of either amphotericin B or terbinafine dilutions. Then, 100 mL of inoculum were transferred to each well. Inocula preparation, time and temperature of incubation, and reading were similar to those described for MIC determination.¹⁰

To evaluate the interaction of agents, the fractional inhibitory concentration index (FICI) was calculated for each combination. Fractional inhibitory concentration (FIC) was calculated for each agent by dividing the inhibitory concentration of each antifungal agent when used in combination by its MIC. FIC values were then added together to define the interaction of the combination. Synergy was defined as an $FICI \leq 0.5$, additive effect when $0.5 < FICI < 1.0$, indifference when $1.0 \leq FICI < 4.0$, and antagonism when $FICI \geq 4.0$.

Broth microdilution MICs of antifungals alone were determined against the isolates before and after 15 days of increased exposure to fluconazole. Geometric means are presented in Table 1. Results demonstrate that, besides fluconazole, resistant derivatives were less susceptible to the rest of antifungals when compared to sensitive strains.

Table 2 depicts the interactions of azoles with terbinafine or amphotericin B by checkerboard method. The majority of combinations had indifferent activity against FS and FR *C. dubliniensis* isolates. However, when interactions were detailed for each isolate, it was possible to note that almost 67% of FS isolates evidenced antagonism for the TRB+ITZ association. Against TRB+VRZ, FS group showed similar percentages for synergy and indifference, but antagonism still remained the most frequent. On the other hand, more positive interactions were obtained when the same azoles were combined with AMB. While 46.67% of isolates presented antagonism against AMB+VRZ, only 20% evidenced the same effect when AMB and VRZ were combined.

Against FR isolates, antagonistic interactions decreased to 20% when TRB and ITZ were combined, whereas synergy and indifference interactions became more prevalent. TRB+VRZ resulted on 53.33% of indifference and synergy decreased

Table 1 - In vitro susceptibilities (mg/mL) of *C. dubliniensis* isolates to antifungal agents before (FS) and after (FR) 15 days of increased exposure to fluconazole

Agents	Group of isolates	Geometric mean	Range	MIC ₅₀	MIC ₉₀
Amphotericin B	FS	0.207	0.060-0.50	0.250	0.500
	FR	0.912	0.250-2.00	1.000	2.000
Fluconazole	FS	0.2145	0.06-0.50	0.125	0.5
	FR	151.47	64-256	128	256
Itraconazole	FS	0.033	0.008-0.25	0.125	0.500
	FR	111.4	32.00-512	4.000	8.000
Terbinafine	FS	0.771	0.060-16.0	0.125	0.250
	FR	157.6	64.00-256	8.000	16.00
Voriconazole	FS	0.006	0.001-0.125	1.000	4.000
	FR	2.047	0.250-16.0	128.0	256.0

Table 2 - Individual analysis of interactions resulted from in vitro antifungal combinations of terbinafine, amphotericin B, itraconazole and voriconazole against fluconazole-susceptible (FS) and -resistant (FR) *C. dubliniensis* isolates

Agents	Group of isolates	Interactions			
		Synergy	Additivity	Indifference	Antagonism
Terbinafine	FS	3.33%	3.33%	26.66%	66.66%
Itraconazole	FR	30%	13.33%	36.66%	20%
Terbinafine	FS	16.66%	10%	16.66%	56.67%
Voriconazole	FR	0%	23.33%	53.33%	23.33%
Amphotericin B	FS	16.66%	20%	40%	23.33%
Itraconazole	FR	13.33%	10%	56.67%	20%
Amphotericin B	FS	6.66%	16.66%	30%	46.67%
Voriconazole	FR	6.66%	60%	23.33%	6.66%

to zero. However, when VRZ was combined with AMB, 60% of isolates showed an additive effect. Against AMB+ ITZ, 13.33% of synergy was obtained, but indifference was the most frequent (56.67%).

The increased use of antifungal agents may have contributed to the development of highly resistant microorganisms or those which are more prone to develop resistance to drugs such fluconazole.¹¹ Although most *C. dubliniensis* clinical isolates are susceptible to azole derivatives, resistant strains have been recovered from oral cavities of human immunodeficiency virus (HIV)-infected patients with oropharyngeal candidiasis and prior exposure to fluconazole.^{8,12} Thus, combining antifungal agents has been suggested as an alternative strategy, and the use of terbinafine in combination with azoles has been suggested as a potential therapeutic option.^{4-6,13}

Despite promising results obtained through some antifungal associations against *Candida* spp., only the combination of caspofungin with terbinafine has already been tested against *C. dubliniensis*.¹⁴ Differential effects were detected when *C. albicans* and *C. dubliniensis* were exposed to the same antifungal combination; it is acceptable the evidence of negative effects against *C. dubliniensis*. The combination of caspofungin and terbinafine appears to result in positive interactive effects against *C. albicans*, whereas against *C. dubliniensis* it did not.¹⁴

In this study, we investigated in vitro interactions of amphotericin B and terbinafine with azoles against FS and FR groups of *C. dubliniensis*. Against the FS group, the best activity was obtained for the combination of amphotericin B with voriconazole, which showed 6.66% of synergistic effect and 60% of additive effect. Although the best synergy rates (16.66%) were obtained for both TRB+VRZ and AMB+ITZ, the latter seemed to be better since it showed half of the antagonism seen with the first. Finally, because 66.66% of isolates evidenced antagonism when terbinafine and itraconazole were combined, it was considered the worst association. Synergy rates (16.66%) were low when compared with results obtained by Perea et al.,¹⁵ Barchiesi et al.,⁴ Cantón et al.,⁵ and Weig & Muller,⁶ who found synergistic or additive interactions when terbinafine and azoles were combined against *C. albicans* strains.

Against the FR group, TRB+ITZ was the association that most frequently resulted in synergy (30%). When terbinafine

was combined with voriconazole, synergy decreased to zero and indifference became the most frequent (53.33%) result. AMB+ITZ resulted in indifference in 56.67% of interactions. But when amphotericin was combined with voriconazole, an additive effect was the most frequent result (60%).

Although the majority of reports mention antagonism between amphotericin B and azoles, data from in vitro studies remain controversial, and indifferent and additive inter-actions are also described.^{3,16} Here, combinations of amphotericin B and azoles mostly resulted in indifferent interactions against *C. dubliniensis* in both groups of isolates.

Among the combinations tested, terbinafine with itraconazole deserves more attention because this association has shown disparate activities: it was the worst combination for FS group (66.6% of antagonism) and the best combination for FR group (30%). On the other hand, on previous studies, terbinafine has improved the in vitro activity of fluconazole and itraconazole against FS or FR *C. albicans* isolates. The combination was also very synergistic against clinical isolates of *Candida glabrata* and no antagonism was detected.¹⁵

In fact, susceptibility of *C. dubliniensis* to antifungal associations was disappointing and frightening if we consider those interactions obtained on previous studies when *C. albicans* strains were tested. If we consider clinical situations in which the resistance phenomenon is emergent, careful identification of such *Candida* species and determination of fluconazole susceptibility are necessary before practitioners choose to treat many mycotic infections with these combinations. Since checkerboard method is a preliminary in vitro test, further studies are required in order to provide clearer information about susceptibility differences between these species when exposed to azole-polyene or azole-allylamine combination.

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Conflict of interest

All authors declare to have no conflict of interest.

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