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In Vitro Study of *Candida tropicalis* Isolates Exhibiting Paradoxical Growth in the Presence of High Concentrations of Caspofungin▽

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Paradoxical growth was noted in RPMI 1640 and antibiotic medium 3 in the case of 14 and 1 of 15 *Candida tropicalis* strains, respectively, at a caspofungin concentration of 12.5 μg/ml using minimum fungicidal concentration tests. Time-kill assays showed that against isolates killed at lower concentrations, caspofungin at a concentration of 12.5 μg/ml was only fungistatic.

The growing body of data shows that some *Candida* strains, which are inhibited by a low concentration of echinocandin antifungals, exhibit growth in the presence of high concentrations of the drugs (2, 9, 10). The frequencies of this paradoxical growth (PG) were found to vary (observed for 10 to 90% of strains) among the most frequently isolated *Candida* species (2, 7, 9). The only species found to grow in the presence of a high concentration of all three marketed echinocandins, i.e., caspofungin (CAS), micafungin (MICA), and anidulafungin, was *C. tropicalis* (2).

Our aim was to examine the occurrence of PG in clinical *C. tropicalis* isolates in vitro by means of MIC and minimum fungicidal concentration (MFC) tests as well as by determining the killing dynamics in time-kill experiments.

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Fifteen *C. tropicalis* clinical isolates and the *C. tropicalis* strain ATCC 750 were used. The CAS (Merck) MICs and MFCs were determined according to the CLSI (formerly NCCLS) method with RPMI 1640 medium (6) and antibiotic medium 3 (AM3; Fluka), as recommended previously (1). In MFC tests, the starting inoculum was increased 100-fold (to 10^5 CFU/ml) (5). The CAS concentration range was 0.024 to 12.5 μg/ml. CAS MICs were read after 24 h, using the partial inhibition criterion (5). After 24 and 48 h, the entire contents of each well containing drug concentrations above the MIC was plated onto Sabouraud dextrose agar (5).

Time-kill studies were performed following the method described previously (4). CAS concentrations ranged from 1 to 512 times the MIC (0.024 to 12.5 μg/ml). Samples were removed at 0, 2, 4, 8, 12, 24, and 48 h and plated onto Sabouraud dextrose agar. Plates were incubated at 35°C for 48 h, in both the MFC and the time-kill tests, and fungicidal activity was defined as a 99.9% reduction in viable CFU/ml compared to that of the starting inoculum (4, 5).

In another experiment, 1 μg/ml of amphotericin B (AMB) (Sigma), fluconazole (FLC) (Pfizer), or flucytosine (5FC) (Sigma) was added to the test tubes containing 12.5 μg/ml of CAS. All assays were repeated at least twice.

In both the RPMI 1640 medium and AM3, MICs were 0.024 μg/ml for all strains, regardless of the starting inoculum. In RPMI 1640 medium, PG was detected in two cases (isolates 4 and 15) at 12.5 μg/ml CAS, using the standard inoculum. In the case of the elevated starting inoculum, partial growth was detected in all wells. In MFC tests, all isolates except number 7 grew at 12.5 μg/ml (Fig. 1). Six of fifteen and 7 out of 15 isolates also grew at 6.25 μg/ml after 24 and 48 h of incubation, respectively.

In AM3, isolate number 6 showed PG after the high-concentration inoculum was used but not with the standard starting inoculum. In the MFC test, this strain also grew at 6.25 and 12.5 μg/ml. Fifteen strains were killed by concentrations ≤0.09 μg/ml CAS (≤4 times the MIC) after 24 h.

The strains tested in the time-kill experiments together with their AMB, FLC, and 5FC MICs are listed in Table 1; representative kill curves with RPMI 1640 medium are shown in Fig. 2.

TABLE 1. MICs and PG patterns of strains used in this study[a]

Strain	MIC (μg/ml)[c]			PG at 6.25 and 12.5 μg/ml after [d]:	
	AMB	FLC	5FC	24 h	48 h
4	2	0.5	0.12	12.5	Both
5	2	0.25	0.12	Both	Both
6[b]	2	0.25	0.12	Both	Both
7	2	0.25	0.12	None	None
8	2	0.5	0.12	12.5	12.5
13	2	0.5	0.12	12.5	12.5
15	2	0.25	0.12	Both	Both
ATCC 750	1	1	0.12	Both	Both

[a] Strains used in the time-kill experiments are shown with their MICs for AMB, FLC, and 5FC and their PG patterns in MFC tests after 24 and 48 h in RPMI 1640 medium.
[b] This isolate also showed PG in AM3.
[c] MICs were determined according to the CLSI method (6).
[d] Both, PG was observed at both concentrations. None, PG was observed at neither concentration.

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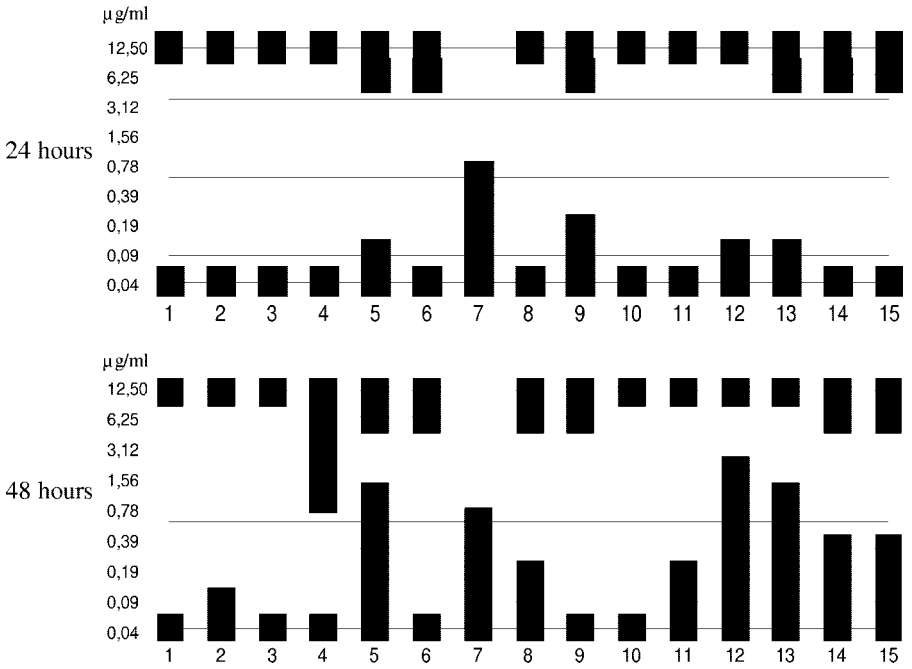

FIG. 1. Growth distribution of the *C. tropicalis* clinical isolates (no. 1 to 15) at different concentrations of CAS (0.4 to 12.50 μg/ml) in the MFC tests after 24 and 48 h in RPMI 1640 medium.

Time-kill curves confirmed the PG of seven of the clinical isolates found to grow paradoxically by using MFC determinations in both media. Ranges of CFU changes varied between −lg0.36 to −lg1.3 and −lg1.51 to +lg0.63 CFU/ml after 24 and 48 h, respectively. Generally, CAS proved to be fungicidal at lower (≤3.12 μg/ml) concentrations, but at high concentrations (6.25 to 12.5 μg/ml), only fungistatic activity was observed (Fig. 2). When we retested the strains growing at 12.5 μg/ml, we obtained essentially the same CAS kill curves.

The killing patterns obtained using the MFC and the time-kill methods (except for isolate 4 and the ATCC 750 strain in RPMI 1640 medium) were identical for the strains tested. *C. tropicalis* ATCC 750 grew at all CAS concentrations tested in the MFC tests, after both 24 and 48 h. In the time-kill test, this strain grew only at 512 times the MIC (12.5 μg/ml), but yeasts were killed at 1 to 256 times the MIC (0.19 to 6.25 μg/ml) for CAS concentrations after 24 h. A similar discrepancy, but to a lesser extent, was found for isolate number 4.

In the time-kill assay, the presence of FLC, but not of AMB or 5FC, at 1 μg/ml, regardless of the medium used, eliminated PG even after 24 h, for all clinical isolates (Fig. 3). In contrast,

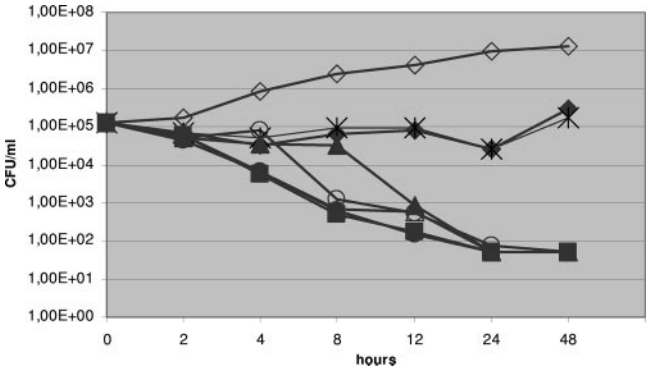

FIG. 2. Representative time-kill plots of *C. tropicalis* isolate number 15 (MIC, 0.024 μg/ml) following exposure to CAS in RPMI 1640 medium. Filled diamonds, 512 times the MIC; asterisks, 256 times the MIC; filled triangles, 128 times the MIC; open circles, 32 times the MIC; filled squares, 8 times the MIC; filled circles, 2 times the MIC; open triangles, 1 times the MIC; open diamonds, drug-free control. Each datum point represents the mean of two independent experiments.

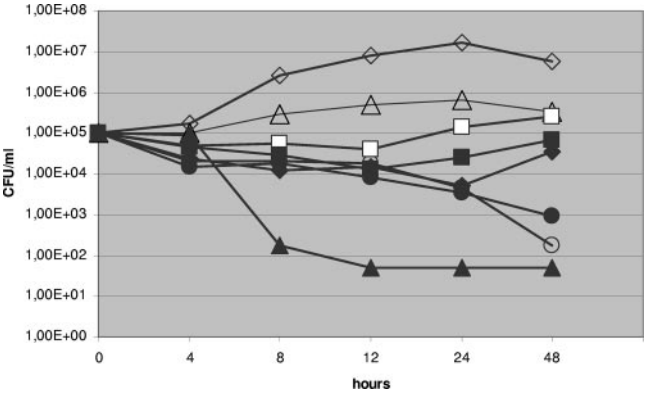

FIG. 3. Time-kill plots of *C. tropicalis* isolate number 4 following exposure to CAS alone and CAS combined with other antifungals in RPMI 1640 medium. Open diamonds, drug-free control; filled diamonds, CAS (12.5 μg/ml) alone; open triangles, FLC (1 μg/ml) alone; filled triangles, CAS (12.5 μg/ml) plus FLC (1 μg/ml); open circles, AMB (1 μg/ml) alone; filled circles, CAS (12.5 μg/ml) plus AMB (1 μg/ml); open squares, 5FC (1 μg/ml) alone; filled squares, CAS (12.5 μg/ml) plus 5FC (1 μg/ml). Each datum point represents the mean of two separate experiments with similar results.

in the case of *C. tropicalis* ATCC 750, the combination of CAS with AMB, but not with FLC or 5FC, eliminated PG.

C. tropicalis is a species that shows PG equally in the presence of CAS, MICA, and anidulafungin (2). Moreover, Pappas et al. observed more treatment failures with daily doses of 150 mg MICA but not with daily doses of 100 mg MICA or 70 mg CAS (8), in cases of patients infected with several *Candida* species including *C. tropicalis*. They suggested that PG may contribute to this phenomenon. Similar in vivo effects were observed for infections by *Aspergillus fumigatus* and *C. albicans* (3, 11).

In our work, visible PG in RPMI 1640 medium was noted in the case of 2/15 (13%) and 14/15 (93%) isolates in the MIC and MFC tests, respectively. Similar to the results reported by Pai et al. (7), we found that the use of AM3 almost totally eliminated this phenomenon; a single isolate showed PG in the MFC tests but none in the MIC tests.

Killing curves obtained with high-concentration CAS clearly indicated that viable *C. tropicalis* cells were present at all times during the time-kill experiment; at very high concentrations, CAS practically became fungistatic rather than fungicidal. The time-kill method was superior to the MFC test for the ATCC strain.

In conclusion, PG could be reliably predicted by the time-kill and MFC tests but not by the MIC test. This may have future implications, because based on these and earlier data (2, 3, 11), the clinical relevance of PG cannot be excluded, suggesting that high doses of echinocandins may lead to treatment failure in certain clinical situations (8).

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