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In Vitro Interaction between Fluconazole and Triclosan against Clinical Isolates of Fluconazole-Resistant *Candida albicans* Determined by Different Methods▽

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The *in vitro* interaction between triclosan and fluconazole against 24 azole-resistant clinical isolates of *Candida albicans* was evaluated by the microdilution checkerboard technique. The synergisms were verified by time-killing curves and agar diffusion tests in selected strains. Antagonistic activity was not detected.

Candida albicans is the primary cause of opportunistic fungal disease in humans. It is predominantly found at low levels among the normal oral flora but can thrive in immunocompromised individuals (16, 25). Fluconazole has been used successfully as a prophylactic and a first-line therapeutic antifungal agent (5, 6, 19). However, the increase in azole use has precipitated a rise in drug resistance in clinical isolates. Triclosan, a chlorinated aromatic compound, has antimicrobial (4, 8, 20), antiparasitic (26), and anti-inflammatory (1, 24) activities. It has been used in personal care products (2). Combination therapy can improve the efficacy of antimicrobial therapy for infections recalcitrant to most treatments. Therefore, we aimed to assess the presence of combination effects with triclosan and fluconazole in *C. albicans*.

A total of 24 clinical isolates of fluconazole-resistant *C. albicans* were used in this study, and *C. albicans* ATCC 10231, *C. parapsilosis* ATCC 90018, and *C. krusei* ATCC 6258 were used as quality controls. The drugs were purchased from Sigma (Sigma-Aldrich).

The drug MICs were determined by broth microdilution according to CLSI method M27-A (3) with an inoculum of 2.5×10^3 CFU/ml. The plates were incubated at 35°C, and the optical density (OD) value was determined at 492 nm after 48 h, a modification to the CLSI reference method. All experiments were conducted in triplicate, and the median MIC-1 endpoint value, which represents an 80% reduction in turbidity, and MIC-2 endpoint value, which represents a 50% reduction in turbidity, were calculated (3). The drug interactions were analyzed using the fractional inhibitory concentration index (FICI) and ΔE models based on the Loewe additivity and Bliss independence theories, respectively (14, 21). The FICIs were defined as the sum of the MICs of each drug used in the combination divided by the MIC of the drug used alone. Synergy and antagonism were defined by FICIs of ≤ 0.5 and >4, respectively (15). The ΔE model was calculated as the sums of the percentages of all statistically significant (SS) synergistic ($\sum$SYN) and antagonistic ($\sum$ANT) interactions. Interactions that were $<100\%$ and $>200\%$ SS interactions were considered weak and strong, respectively. Interactions that were 100 to 200% SS interactions were considered moderate (14). The numbers of SS synergistic and antagonistic combinations were calculated for each strain.

A 100-μl sample of 10^6 CFU/ml *C. albicans* YL345, which exhibited the best synergistic effect, was spread onto a yeast extract-peptone-dextrose agar surface. Subsequently, 6-mm-diameter paper disks, impregnated with drugs or dimethyl sulfoxide (DMSO) alone, were placed onto the surface. The inhibition zones were measured using a dial caliper after a 48-h incubation at 35°C. The tests were performed in duplicate (9, 18).

The time-kill curves were conducted in an RPMI 1640 medium with 10^5 CFU/ml *C. albicans* YL345. At different time points after the drug incubation, 100 μl of the tube contents was subcultured in serial dilutions (10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}) on Sabouraud dextrose agar plates. Colony counts were determined after a 48-h incubation at 35°C. The results were reported as the mean ± standard deviation of all three replicates conducted for each compound, alone and

TABLE 1. Checkerboard analysis of *in vitro* interaction between TCL and FLC against 24 clinical isolates of *C. albicans*[a]

Drug	Median MIC-2 endpoint (range) of drug (μg/ml)		Median MIC-1 endpoint (range) of drug (μg/ml)	
	Alone	In combination	Alone	In combination
FLC	16 (4–32)	1 (1–2)	256 (64–>512)	2 (1–4)
TCL	32 (32–64)	8 (4–8)	64 (32–64)	8 (8–16)

[a] TCL, triclosan; FLC, fluconazole.

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TABLE 2. FICI and ΔE analyses of *in vitro* interaction between TCL and FLC against 24 clinical isolates of *C. albicans*[a]

Endpoint type	Result according to nonparametric method[b]				
	FICI model		ΔE model		
	FICI, median (range)	INT	ΣSYN % (*n*)	ΣANT % (*n*)	INT
MIC-2	0.313 (0.125 to 0.375)	SYN (all isolates)	116.8 (15) to 589.2 (22)	−28.3 (5) to −95.2 (12)	SYN (21 isolates), IND (3 isolates)
MIC-1	0.25 (0.125 to 0.25)	SYN (all isolates)	50.1 (8) to 686.8 (24)	−8.3 (2) to −91.9 (7)	SYN (22 isolates), IND (2 isolates)

[a] TCL, triclosan; FLC, fluconazole.
[b] INT, interpretation; SYN, synergism; ANT, antagonism; IND, indifference; *n*, number of interactions.

in combination. The synergism and antagonism were defined as respective increases or decreases of ≥ 2 $\log_{10}$ CFU/ml in antifungal activity produced by the drug combination compared with the more active agent alone after 24 h (10, 12).

The checkerboard results are summarized in Table 1. The MIC-2 endpoint values for fluconazole and triclosan in *C. albicans* ranged from 4 to 32 μg/ml and from 32 to 64 μg/ml, respectively. The drug combination markedly reduced the MIC-2 endpoints of fluconazole and triclosan to 1 to 2 μg/ml and 4 to 8 μg/ml, respectively. A previous report has stated that the *in vivo* triclosan concentration in saliva was about 13 μg/ml at 10 min after brushing with toothpaste, and the duration of activity of triclosan at a concentration of 10 μg/ml in saliva was about 0.7 h (13). From our data, the

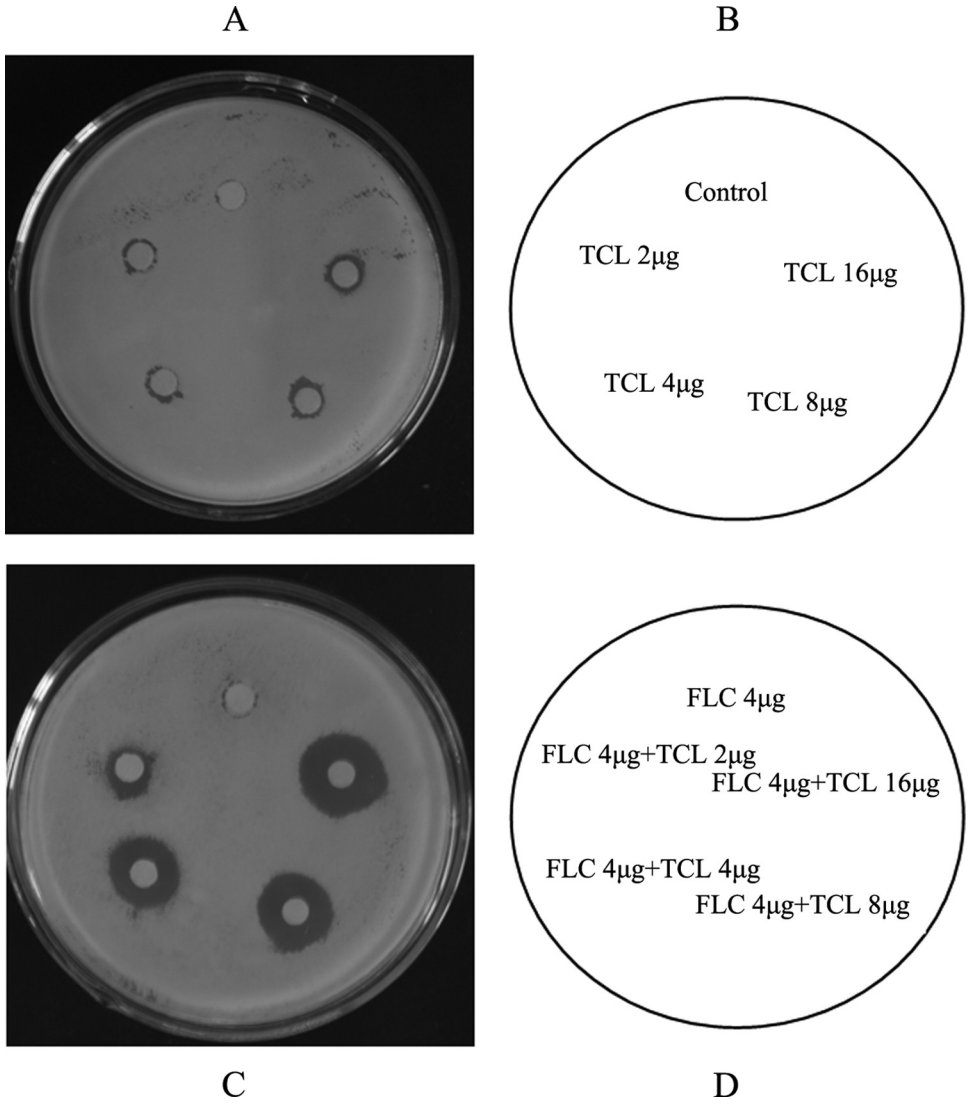

FIG. 1. Agar disk diffusion assay for FLC combined with TCL in *C. albicans* YL345. Panel B describes the image for panel A, and panel D describes the image for panel C.

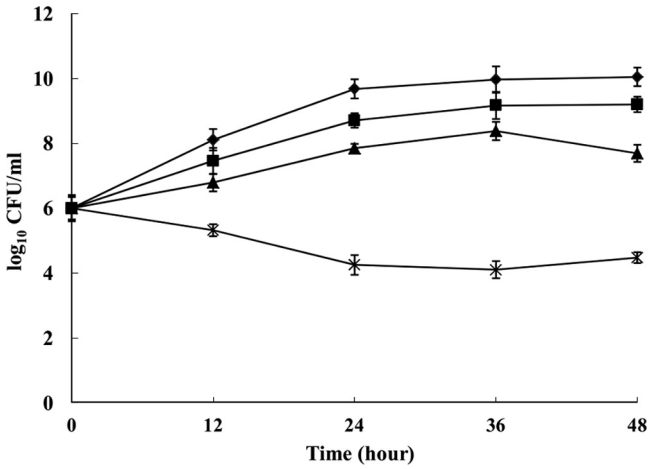

FIG. 2. Time-kill curves for fluconazole (FLC) and triclosan (TCL) alone and in combination in clinical azole-resistant *C. albicans* YL345. ◆, growth control; ■, FLC; ▲, TCL; ×, FLC plus TCL.

MIC-2 values of triclosan against azole-resistant *C. albicans* strains were 4 to 8 μg/ml when it was combined with fluconazole. A concentration of triclosan in saliva between 10 and 13 μg/ml was adequate to inhibit an azole-resistant strain when the two drugs were combined.

The corresponding median FICI and Δ*E* values are shown in Table 2. The FICIs ranged from 0.125 to 0.375 and from 0.125 to 0.25 when analyzed using the MIC-2 and the MIC-1 endpoints, respectively. The Δ*E* values ranged from 116.8% to 589.2% when calculated using the MIC-2 endpoint. Antagonisms were not observed.

The synergism between fluconazole and triclosan was confirmed by agar diffusion tests (Fig. 1). The halo diameters produced by the combination were predominantly larger than ones produced by single-drug treatments. The sizes of the inhibition zones increased to 19.2, 18, 15.6, and 10.8 mm when 16 μg/ml fluconazole was combined with 16, 8, 4, and 2 μg/ml of triclosan, respectively.

The time-kill curves verified the synergic combinations (Fig. 2). Triclosan and fluconazole did not significantly affect isolate growth when the drugs were used alone at 16 μg/ml and 4 μg/ml, respectively. The combination therapy yielded a 3.0-$\log_{10}$-CFU/ml decrease compared with triclosan alone after 24 h, wherein there was a significant difference ($P < 0.01$).

Taken together, our findings indicate that triclosan exhibits an antifungal effect *in vitro* against azole-resistant *C. albicans* when combined with fluconazole. In the checkerboard assay, the FICI model has been frequently used to determine the interaction between antifungal drugs (7, 9, 12, 17, 21, 23). The Δ*E* model is a useful method for characterizing drug interactions. We verified the positive interactions using the agar diffusion test and time-kill curves, which were able to detect differences in the rate and degree of antifungal activity over time (11). An agar diffusion test can provide more visually convincing results. A combination treatment with triclosan has been previously demonstrated to significantly enhance the efficacy of triclosan against microbes (20, 22). In contrast to various previous reports (20, 22), triclosan is a better synergist to fluconazole against *C. albicans.*

In conclusion, the combination treatment of fluconazole and triclosan effectively synergizes against *C. albicans.* Our findings may provide an alternative approach to overcoming antifungal drug resistance. However, the mechanisms underlying the synergy must be further elucidated.

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