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Multilaboratory Testing of Antifungal Combinations against a Quality Control Isolate of *Candida krusei*▽†

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***Candida krusei* ATCC 6258 was tested by eight laboratories using 96-well plates containing checkerboard pairwise combinations of amphotericin B (AMB), posaconazole (PSC), caspofungin (CSP), and voriconazole (VRC). The methodology led to reproducible results across the laboratories. All drug combinations yielded MICs lower than the MICs of any two drugs tested singly, and combinations of AMB, PSC, CSP, and VRC were indifferent (no antagonism) by summations of fractional inhibitory concentration.**

Several new antifungal agents (caspofungin [CSP], posaconazole [PSC], and voriconazole [VRC]) have recently become available for the treatment of deeply invasive fungal infections. These drugs are reported to vary in terms of efficacy, bioavailability, and tissue penetration in infections caused by pathogenic molds and yeasts (6, 17). They have been tested in the laboratory against a wide range of pathogenic fungi to determine their in vitro efficacy even though interpretive breakpoints have been established only for a few pathogenic yeasts (3). There is a recent trend to use the newer drugs in combination with or after more established antifungal therapy. Clinical and laboratory studies have evaluated the interactions among the agents of the classes of polyenes, triazoles, echinocandins, and allylamines in vitro, in vivo, and in clinical trials (13, 15). Additionally, there are several reports on the compassionate use of antifungal combinations for the treatment of recalcitrant infections caused by common as well as rare pathogens (4, 14, 18). However, seldom are the isolates from patients tested in vitro beforehand for interactions in the laboratory to evaluate the efficacy of these combinations. Such tests for drug interactions might be helpful in the choice of combination therapy, especially in view of the high cost of new drugs and the potential for antagonistic interactions.

A number of laboratories have reported the in vitro testing of antifungal agents in combination (1, 8, 16). Most of the studies were based on checkerboard titrations using 96-well plates although the time-kill assay and Etest have also been tested. Each one of the aforementioned methods has inherent advantages and limitations for antifungal interaction studies (2, 9). The literature on multilaboratory evaluations of antifungal combinations is limited, and standardized and recommended methods have yet to emerge. The present report describes a pilot program designed to test antifungal combinations at eight participant sites, with the long-term objective of identifying a consensus method or methods suitable for routine use in the clinical laboratory.

The drug combinations were studied by means of a two-dimensional broth microdilution checkerboard procedure using two-antifungal agents as described in the Clinical Microbiology Procedures Handbook (10). The 96-well plates were commercially prepared (Trek Diagnostics Systems., Cleveland, OH) according to the CLSI M27-A2 reference method (11) using checkerboard combinations. The drugs used were amphotericin B (AMB; Sigma Chemical Co.), CSP (Merck, Inc.), PSC (Schering-Plough Corp.), and VRC (Pfizer, Inc.). Drug concentration ranges used were as follows: AMB, 0.015 to 4.0 μg/ml; CSP, 0.03 to 2.0 μg/ml; PSC, 0.008 to 0.5 μg/ml; and VRC, 0.008 to 0.5 μg/ml. The 96-well grids used to accommodate various drugs alone or in combinations (final volume of drugs diluted in RPMI 1640 broth, 100 μl) are shown in Fig. S1 in the supplemental material. The plates were shipped frozen by the manufacturer to the eight participating laboratories. RPMI 1640 broth for drug dilutions was also shipped. The plates were stored at −70°C until use. *Candida krusei* ATCC 6258, a quality control strain described in the CLSI method (11), was used for testing of various antifungal combinations. Each participating laboratory used an isolate of this strain from its own collection. The broth microdilution test was performed in accordance with the M27-A2 reference method (11). The 96-well plates were thawed as required. Inoculum was prepared from 18- to 24-h-old culture on Sabouraud dextrose agar plates. The inoculum was adjusted to 0.5 McFarland standards, using a spectrophotometer at a 530-nm wavelength. Ten microliters of the above inoculum was added to 11 ml of RPMI broth, and 100 μl was dispensed into microtiter wells to equal a final volume of 200 μl. The mixture of drugs and inoculum

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TABLE 1. Summary of MICs of various antifungal agents alone and in combination against *C. krusei* ATCC 6258, as reported by the eight participating laboratories[a]

Antifungal agent(s)	MIC (μg/ml)		MIC_{50} (drug A/drug B [μg/ml])		MIC_{100} (drug A/drug B [μg/ml])	
	Range	Mode	Range	Mode	Range	Mode
AMB	1.0–2.0	1				
PSC	0.12–0.5	0.25				
CSP	0.25–1.0	0.5				
VRC	0.12–0.5	0.25				
AMB+ PSC			0.06–1.0/0.008–0.5	0.5/0.06	0.25–1.0/0.008–0.25	1.0/0.25
AMB+ CSP			0.015–1.0/0.03–1.0	0.5/0.25	0.12–1.0/0.03–1.0	0.5/0.5
AMB+VRC			0.015–1.0/0.008–0.25	1/0.008	2.0/0.008–0.5	2.0/0.008
PSC + CSP			0.008–0.25/0.03–0.25	0.25/0.12	0.008–0.25/0.5–1.0	0.008/1.0
PSC +VRC			0.008–0.25/0.008–0.25	0.008/0.25	0.12–0.5/0.008–0.5	0.5/0.5
VRC+ CSP			0.008–0.25/0.03–0.5	0.008/0.25	0.008–0.06/1.0–2.0	0.008/1.0

[a] Values are based on 10 replicate determinations.

was incubated at 35°C and was read after 48 h. MICs of individual antifungal agents correspond to either complete (100% for AMB) or prominent (50% for PSC, VRC, or CSP) yeast growth inhibition (decrease in turbidity) compared to growth in the control well. For the wells with a combination of drugs, growth was scored on a scale of 0 to 4+. An image of a plate illustrating the scale used by the participating laboratories to ensure uniform reading is shown in Fig. S2 in the supplemental material. According to this scale, growth was evaluated as follows: 0, optically clear; 1+, 25% growth compared to control; 2+, 50% growth compared to control, 3+, 75% growth compared to control; and 4+, growth equal to that in the control well. MICs of drug combinations correspond to prominent growth inhibition (2+, or MIC_{50}) because a majority of drugs tested (azoles and echinocandin) are usually read at this cutoff. In addition to the MIC_{50} of drug combinations, we also calculated the MIC_{100} to further determine if the combined drug effects were influenced by the reading cutoff. Drug combination interactions were calculated algebraically by determining the fractional inhibitory concentration (FIC) as detailed in the Clinical Microbiology Procedures Handbook (10). FIC_A is calculated as the MIC of drug A in combination/MIC of drug A alone and FIC_B equals the MIC of drug B in combination/MIC of drug B alone. The summation (Σ) of FIC was calculated as follows: $\Sigma FIC = FIC_A + FIC_B$. The interpretation of ΣFIC was as follows: ≤ 0.5, synergistic; >0.5 to <4.0, indifferent (no antagonism); ≥ 4.0, antagonistic. The eight participating laboratories conducted 10 replicate tests for each individual drug and for each drug combination. The results were submitted electronically to the laboratory at the Wadsworth Center and analyzed using SigmaPlot and SigmaStat software (Systat Software, Inc., San Jose, CA). ΣFIC values were analyzed and expressed as mode and ranges for each combination, and variance among laboratories for MICs and for ΣFIC values was determined by the coefficient of variation (CV).

A total of 1,920 MICs were reported by the eight participating laboratories for six antifungal combinations tested using a total of 480 plates (Table 1). The MICs for the agents tested alone showed excellent agreement with the reported CLSI quality control ranges (AMB, 100%; PSC, 99%; CSP, 100%; VRC, 99%). In two instances, two different laboratories reported 1 out of 10 readings (PSC and VRC) outside of the reference range; these values were not considered in final calculations. Most drug combinations were within a 1-dilution difference between the MIC_{50} and MIC_{100} except the combination of PSC and CSP (Table 1). Furthermore, all drug combinations yielded MICs lower than the MIC of either of the drugs tested singly. The lower MICs of the combinations were obtained with concentrations one-half to one-third lower than concentrations of the individual drugs. Thus, there was strong empirical evidence for synergistic activities of the antifungal combinations tested. We further evaluated this inference by calculating the ΣFICs for the various combinations (Table 2). The median ΣFICs using the MIC_{50} of the combinations were as follows: AMB with PSC, 0.88; AMB with CSP, 0.92; AMB with VRC, 0.78; PSC with CSP, 0.68; PSC with VRC, 0.90; and VRC with CSP, 0.90. The percentage CV ranged from 13.2% to 34.75%. These derivative values did not satisfy the recommended cutoff of ≤ 0.5 for defining true antifungal synergy (5,

TABLE 2. ΣFICs for pairwise combinations of antifungal agents tested against *C. krusei* ATCC 6258 suggestive of indifferent (no antagonism) interactions

Antifungal agents	ΣFIC of the combination from laboratory:								Median ΣFIC (range)	CV (%)[a]
	1	2	3	4	5	6	7	8		
AMB+ PSC	0.52	1.10	0.76	1.03	0.96	0.72	0.80	1.08	0.88 (0.52–1.10)	22.56
AMB+ CSP	1.04	0.93	0.84	0.92	1.14	1.11	0.63	0.84	0.92 (0.63–1.14)	17.63
AMB+VRC	0.69	0.91	0.63	0.83	0.61	0.79	0.78	1.29	0.78 (0.61–1.29)	25.35
PSC + CSP	0.98	0.55	0.63	0.72	0.79	1.08	0.65	0.29	0.68 (0.29–1.08)	34.75
PSC +VRC	0.91	0.95	0.99	0.89	0.95	0.85	0.90	0.62	0.90 (0.62–0.99)	13.2
VRC+ CSP	0.95	0.93	0.84	1.19	0.99	1.32	0.90	0.57	0.90 (0.57–1.32)	23.4

[a] CV was calculated as follows: (standard deviation/mean) × 100.

10). Similar values were obtained when the median ΣFICs were calculated using the MIC_{100}s of drug combinations (data not shown). Thus, the pairwise combinations of AMB, PSC, CSP, and VRC were indifferent (no antagonism) for the reference strain. Further examination of the data from the eight participating laboratories revealed that most of the replicate test results fell within a narrow range even though some outlier readings were reported by all participants except laboratory 3 (see Fig. S3 in the supplemental material). Overall, the observed narrow range of intra- and interlaboratory results for single drugs and for two-drug combinations reaffirmed the excellent predictability of *C. krusei* ATCC 6258 in the M27-A2 method (11). We believe that consistent endpoint recording on the 0-to-4+ scale was facilitated by the provision of a visual image guide to each laboratory before the start of the study. This was important, considering that the drugs tested in this study had different modes of actions. The absence of true synergistic activity (ΣFIC of ≤0.5) seen for any of the drug combinations tested could partially be due to having only one test strain employed in the study, or it could be due to the inherent properties of *C. krusei* ATCC 6258, given that other investigators have previously reported an absence of synergy for this strain in testing with various drug combinations (1, 8).

Many limitations have been ascribed to the checkerboard titration method for drug combination testing. These include lack of time course information for the drugs, simultaneous rather than sequential addition of drugs, use of concentrations of drugs higher than achievable serum levels, and artifacts resulting from FIC interpretations (9, 12). Alternatives such as the time-kill assays have been ascribed superiority, primarily because of their emphasis on the time course of the activity (8, 16). However, the time-kill method may be too labor-intensive and time-consuming to be practical for busy clinical laboratories, aside from other issues such as a fixed inoculum size, the small number of drug concentrations used, and limitations of readings to one time point (7). Etest is another alternative test reported to provide reliable testing of antifungal drug combinations in the laboratory (1). Although a few investigators have conducted head-to-head comparisons of these three methods, concordances among checkerboard, time-kill, and Etest results thus far have been variable (1, 8). Overall, our pilot multilaboratory study expands upon the earlier reports that the M27-A2 method can be used to obtain reproducible results for combinations of antifungal drugs against yeasts (1, 8, 9). Our use of a relatively resistant quality control strain further indicated that potentially any *Candida* strain, including strains resistant to one or more drugs, can be tested reproducibly for in vitro susceptibility to various drug combinations. Such testing in the clinical laboratory could have a bearing on the selection of an appropriate therapy in cases where conventional tests identify an isolate resistant to one or more antifungal agents or where a patient may not be responding to monotherapy. Further multilaboratory studies assessing various *Candida* species are clearly indicated to enable standardization of readings and interpretations of the checkerboard titration test for combinations of antifungal drugs.

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