ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Apr. 2011, p. 1543–1548 Vol. 55, No. 4
0066-4804/11/$12.00 doi:10.1128/AAC.01510-09

Multilaboratory Testing of Two-Drug Combinations of Antifungals against *Candida albicans*, *Candida glabrata*, and *Candida parapsilosis*[▽]

Vishnu Chaturvedi,[1]* Rama Ramani,[1]† David Andes,[2] Daniel J. Diekema,[3] Michael A. Pfaller,[3] Mahmoud A. Ghannoum,[4] Cindy Knapp,[5] Shawn R. Lockhart,[6] Luis Ostrosky-Zeichner,[7] Thomas J. Walsh,[8] Karen Marchillo,[2] Shawn Messer,[4] Amanda R. Welshenbaugh,[4] Cara Bastulli,[5] Noreen Iqbal,[6] Victor L. Paetznick,[7] Jose Rodriguez,[7] and Tin Sein[8]

Mycology Laboratory, Wadsworth Center, New York State Department of Health, Albany, New York[1]; University of Wisconsin, Madison, Wisconsin[2]; University of Iowa, Iowa City, Iowa[3]; Center for Medical Mycology, Case Western Reserve University, Cleveland, Ohio[4]; Trek Diagnostic Systems, Cleveland, Ohio[5]; Centers for Disease Control and Prevention, Atlanta, Georgia[6]; University of Texas Medical School at Houston, Houston, Texas[7]; and National Cancer Institute, Bethesda, Maryland[8]

Received 26 October 2009/Returned for modification 3 January 2010/Accepted 18 January 2011

There are few multilaboratory studies of antifungal combination testing to suggest a format for use in clinical laboratories. In the present study, eight laboratories tested quality control (QC) strain *Candida parapsilosis* ATCC 22019 and clinical isolates *Candida albicans* 20533.043, *C. albicans* 20464.007, *Candida glabrata* 20205.075, and *C. parapsilosis* 20580.070. The clinical isolates had relatively high azole and echinocandin MICs. A modified CLSI M27-A3 protocol was used, with 96-well custom-made plates containing checkerboard pairwise combinations of amphotericin B (AMB), anidulafungin (AND), caspofungin (CSP), micafungin (MCF), posaconazole (PSC), and voriconazole (VRC). The endpoints were scored visually and on a spectrophotometer or enzyme-linked immunosorbent assay (ELISA) reader for 50% growth reduction (50% inhibitory concentration [IC_{50}]). Combination IC_{50}s were used to calculate summation fractional inhibitory concentration indices (FICIs) (ΣFIC) based on the Lowe additivity formula. The results revealed that the IC_{50}s of all drug combinations were lower or equal to the IC_{50} of individual drugs in the combination. A majority of the ΣFIC values were indifferent (ΣFIC = 0.51 to 2.0), but no antagonism was observed (ΣFIC ≥ 4). Synergistic combinations (ΣFIC ≤ 0.5) were found for AMB-PSC against *C. glabrata* and for AMB-AND and AMB-CSP against *C. parapsilosis* by both visual and spectrophotometric readings. Additional synergistic interactions were revealed by either of the two endpoints for AMB-AND, AMB-CSP, AMB-MCF, AMB-PSC, AMB-VRC, AND-PSC, CSP-MCF, and CSP-PSC. The percent agreements among participating laboratories ranged from 37.5% (lowest) for AND-CSP and POS-VOR to 87.5% (highest) for AMB-MCF and AND-CSP. Median ΣFIC values showed a wide dispersion, and interlaboratory agreements were less than 85% in most instances. Additional studies are needed to improve the interlaboratory reproducibility of antifungal combination testing.

A number of newly available antifungal drugs for the treatment of serious fungal infections have relatively different efficacies, bioavailabilities, and tissue penetrations (1, 2, 5, 23). These drugs have been tested in the laboratory against a wide range of pathogenic fungi to determine their therapeutic efficacy, and interpretive breakpoints have been established or proposed for a few pathogenic yeasts (20, 41, 42). Current practices are evolving to use the newer drugs in combinations either with or after a more established therapeutic regimen for the treatment of serious yeast infections (18, 26, 35, 37, 43, 50). Potential benefits of using these antifungal drugs in combinations against serious infections include a broader spectrum, reduced toxicity, lower likelihood of the emergence of acquired resistance, and synergistic or additive interactions (7, 15, 19, 24, 28, 33).

* Corresponding author. Mailing address: Mycology Laboratory, Wadsworth Center, New York State Department of Health, 120 New Scotland Ave., Albany, NY 12208. Phone: (518) 474-4177. Fax: (518) 486-7971. E-mail: vishnu@wadsworth.org.

† Present address: Laboratory for Viral Diseases, Wadsworth Center, New York State Department of Health, Albany, NY.

▽ Published ahead of print on 31 January 2011.

Previously reported studies have documented interactions among the agents of the classes of polyenes, triazoles, echinocandins, and allylamines *in vitro*, *in vivo*, and in clinical trials against *Candida* spp. and candidiasis (35, 37, 43, 48). Additionally, there are several reports of the use of antifungal combinations for the treatment of recalcitrant infections caused by yeast pathogens (17, 30, 46). However, seldom are the isolates from patients tested in clinical laboratories in order to evaluate the possible efficacy of these combinations in clinical care. Such tests for drug interactions could provide valuable guidance for the selection of combination therapy (20, 27).

Various standardized methods and FDA-approved commercial devices are now available for antifungal susceptibility testing. These include the CLSI (Clinical and Laboratory Standards Institute; formerly NCCLS) method, the EUCAST (European Committee on Antimicrobial Susceptibility Testing) method, and YeastOne, Etest, and disk diffusion for rapid testing of pathogenic yeasts (6, 10, 13, 14). All of the above-mentioned methods and devices either evolved from or were standardized through the multilaboratory testing of the variables encountered in antifungal susceptibility tests. A number of laboratories have also reported results for the *in vitro* testing

TABLE 1. Antifungal MICs obtained by visual scoring for one QC strain and four clinical strains of *Candida* species by eight participating laboratories

Antifungal drug	Median IC_{50} (μg/ml) (range)				
	C. parapsilosis ATCC 22019	*C. albicans* 20533.043	*C. albicans* 20464.007	*C. glabrata* 20205.075	*C. parapsilosis* 20580.070
AMB	1.0 (0.5–2.0)	1.0 (0.5–2.0)	1.0 (0.5–2.0)	1.0 (0.5–2.0)	1.0 (0.5–4.0)
AND	2.0 (0.5–2.0)	0.06 (0.03–0.06)	0.25 (0.12–0.5)	0.06 (0.03–0.06)	2.0 (2.0–4.0)
CSP	1.0 (0.5–2.0)	0.12 (0.06–1.0)	1.0 (1.0–2.0)	0.06 (0.06–0.12)	1.0 (0.5–1.0)
MCF	2.0 (1.0–4.0)	0.06 (0.03–0.12)	0.12 (0.12–0.5)	0.06 (0.06–0.5)	2.0 (1.0–2.0)
PSC	0.25 (0.12–0.5)	1.0 (0.5–2.0)	0.5 (0.25–1.0)	2.0	0.12 (0.06–0.25)
VRC	0.03 (0.03–0.12)	2.0 (1.0–2.0)	2.0	2.0	0.015

of antifungal agents used in combination. Most of the studies were based on checkerboard dilutions and calculations of fractional inhibitory concentration (FIC) values, although the time-kill assay, the Etest, and side-by-side comparisons of multiple methods have also been reported (8, 9, 11, 29, 34, 38, 44, 49). Each one of the above-mentioned methods and devices has advantages and limitations for the verification of antifungal interactions in two-drug combinations (15, 32, 36). The literature on multilaboratory evaluations of antifungal combinations is limited, and standardized methods have yet to emerge. The present report extends our previous report on the multilaboratory testing of antifungal combinations (12). This study tested five fungal strains, including clinical isolates, and six antifungal agents in 15 possible combinations. The long-term goal of our investigations is to develop a standardized antifungal combination testing method for routine use in clinical laboratories.

MATERIALS AND METHODS

Antifungal agents and fungal strains. Ninety-six-well plates were commercially prepared (Sensititre custom plates; Trek Diagnostics Systems, Cleveland, OH). The plates contained antifungal drugs alone and in checkerboard dilutions of two-drug combinations using concentration ranges slightly modified from those recommended by CLSI document M27-A3 (13, 14). The drugs used were amphotericin B (AMB; Sigma Chemical Co., St. Louis, MO) (range, 0.06 to 4.0 μg/ml), posaconazole (PSC; Schering-Plough Corp., Kenilworth, NJ) (range, 0.015 to 1.0 μg/ml), voriconazole (VRC) (range, 0.015 to 1.0 μg/ml), anidulafungin (AND; Pfizer Inc., Groton, CT) (range, 0.03 to 2.0 μg/ml), caspofungin (CSP; Merck & Co., Rahway, NJ) (range, 0.06 to 4.0 μg/ml), and micafungin (MCF; Astellas Pharma Inc., Tokyo, Japan) (range, 0.06 to 4.0 μg/ml). Single drugs or drug combinations were dispensed into individual wells in 100-μl final volumes. The manufactured plates were shipped frozen by the vendor to the eight participating laboratories. RPMI 1640 broth for drug dilutions was also shipped. The plates were stored at −70°C until further use. *Candida parapsilosis* ATCC 22019, a quality control (QC) strain, was described previously (13, 14). Each participating laboratory used this QC strain from its own collection. Four clinical strains with relatively high azole and echinocandin MICs, namely, *Candida albicans* 20533.043, *C. albicans* 20464.007, *C. glabrata* 20205.075, and *C. parapsilosis* 20580.070, were obtained from the Department of Pathology, University of Iowa. These strains were described previously (39, 40).

MIC determinations. Briefly, custom 96-well plates were thawed as required. An inoculum was prepared from 18- to 24-h-old cultures at 35°C on Sabouraud dextrose agar plates. The inoculum was adjusted to a 0.5 McFarland standard using a spectrophotometer at a 530-nm wavelength. Twenty milliliters of the inoculum was added to 11 ml of RPMI broth, and 100 μl was dispensed into microtiter wells, to give a final volume of 200 μl. The mixture of drugs and inocula was incubated at 35°C and was read after 24 h and 48 h. MICs for yeasts tested with a single drug corresponded to either complete (100% for AMB) or prominent (50% for PSC, VRC, AND, MCF, or CSP) growth inhibition (decrease in turbidity) compared to growth in the control well (13, 14).

Combination studies. At 24 h, preliminary MIC readings were collected; however, the data were not used for further analyses, because although the endpoint readings for echinocandins are obtained at 24 h, for azoles and amphotericin B, 48-h readings are recommended. After 48 h, the areas of the plates that tested the drug combinations were scored on a scale of 0 to 4, where 0 denotes optically clear, 1+ indicates 25% growth relative to the control, 2+ indicates 50% growth relative to the control (50% inhibitory concentration [IC_{50}]), 3 indicates 75% growth relative to the control, and 4+ indicates growth equal to that in the control well (12). An image of a 96-well plate illustrating wells with the 0-to-4 scale readings was circulated to the participating laboratories as a reference to ensure uniform readings. The calculation of summation fractional inhibitory concentration indices (FICIs) (ΣFICs) was done on the basis of the Loewe additivity formula (12, 32, 49). Plates were also read with a spectrophotometer or an enzyme-linked immunosorbent assay (ELISA) reader. The contents of the wells were pipetted up and down 5 to 6 times before being transferred onto a new 96-well plate and read at 550 nm. These readings were used to assess percent growth by the following formula: percent growth = (optical density at 550 nm [OD_{550}] of a well − background OD_{550} of this well)/(OD_{550} of the drug-free well − background OD_{550} of the drug-free well) × 100%.

A 50% reduction in growth was considered to be the IC_{50}, similarly to visual scoring. Each participating site repeated all tests once. The visual and spectrophotometric readings were recorded on Microsoft Excel worksheets and then transferred electronically or as printed copies to the Wadsworth Center. Drug combination interactions were calculated algebraically by determining the FIC as detailed in the *Clinical Microbiology Procedures Handbook* (3). FIC_A equals 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 ΣFIC was determined as follows: $\Sigma FIC = FIC_A + FIC_B$. The interpretation of ΣFIC values was as follows: ≤0.5, synergistic; >0.5 to <4.0, indifferent (no antagonism); and ≥4.0, antagonistic (12, 32, 49). ΣFIC values were analyzed and expressed as medians and ranges for each combination. Percent agreements among the eight laboratory readings were expressed as percent agreements in terms of readings as synergy (FICI ≤ 0.50) and as indifferent (FICI = 0.51 to ≤4.00). A cutoff of ≥85% agreement was considered significant (16). For percent agreement, ΣFIC values determined by the eight participating laboratories (seven for spectrophotometric readings) for each antifungal combination and for each strain tested were taken into consideration. Agreement was defined as the percentage of ΣFIC results within a value of ±0.02 among the laboratories. For percent agreement, the most frequent concordance among the ΣFIC values determined by the participating laboratories for both the visual and the spectrophotometric readings for each antifungal combination and strain were calculated and converted into a percentage. For example, among eight participating laboratories, for the AMB-MCF combination, 7 laboratories reported an ΣFIC of 0.50, and one laboratory reported an ΣFIC of 0.75; the percent agreement would be as follows: 7/8 × 100 = 87.5. Correlation coefficients (CCs) were also calculated by using Pearson's formula with a 95% confidence interval (CI). Pearson's correlation coefficients ranged from −1.00 to +1.00. A correlation coefficient of +1.00 indicates that as values for one variable increase, there is a perfectly predictable increase in values for the other variable. Similarly, a correlation coefficient of −1.00 indicates that as values for one variable increase, there is a perfectly predictable decrease in values for the other variable. In the present study, no negative correlations were observed. The following criteria were used to classify the strength of the correlation: 0.0 to 0.2 for a very weak correlation; 0.2 to 0.4 for a low correlation (not very significant); 0.4 to 0.7 for a moderate correlation; 0.7 to 0.9 for a strong, high correlation; and 0.9 to 1.0 for a very strong correlation. All ΣFIC values were included in the analysis. This statistical analyses were performed by using Systat software (Systat Software Inc., Chicago, IL).

TABLE 2. Median ΣFICs of antifungal drug combinations for five *Candida* strains calculated from visual readings reported by eight participating laboratories

Antifungal drug combination	Median ΣFIC (% agreement/correlation coefficient)				
	C. parapsilosis (ATCC 22019)	*C. albicans* 20533.043	*C. albicans* 20464.007	*C. glabrata* 20205.075	*C. parapsilosis* 20580.070
AMB-AND	0.56 (37.5/0.42)	0.79 (75.0/0.82)	0.56 (62.5/0.88)	0.84 (75.0/0.86)	0.38 (75.0/0.62)
AMB-CSP	0.62 (62.5/0.54)	0.74 (37.5/0.29)	0.53 (75.0/0.85)	1.18 (62.5/0.65)	0.49 (62.5/0.56)
AMB-MCF	0.51 (87.5/0.96)	1.06 (37.5/0.48)	0.53 ((50.0/0.67)	1.06 (87.5/0.91)	0.56 (50.0/0.69)
AMB-PSC	0.74 (37.5/0.18)	0.59 (75.0/0.51)	0.53 (87.5/0.97)	0.46 (37.5/0.32)	0.50 (87.5/0.95)
AMB-VRC	1.045 (0.0/0.48)	0.51 (87.5/0.91)	0.57 (50.0/0.64)	0.63 (37.5/0.31)	1.06 (37.5/0.29)
AND-CSP	0.56 (87.5/0.86)	1.00 (75.0/0.68)	0.56 (25.0/0.26)	1.75 (87.5/0.91)	0.78 (37.5/0.42)
AND-MCF	0.62 (75.0/0.63)	1.75 (75.0/0.41)	0.74 (75.0/0.89)	1.5 (75.0/0.68)	0.84 (50.0/0.48)
AND-PSC	0.51 (37.5/0.29)	0.81 (37.5/0.18)	0.49 (37.5/0.44)	0.76 (62.5/0.57)	0.50 (50.0/0.30)
AND-VRC	1.06 (25.0/0.18)	0.79 (50.0/0.46)	0.80 (37.5/0.29)	0.76 (87.5/0.82)	1.01 (37.5/0.34)
CSP-MCF	0.65 (87.5/0.90)	1.31 (50.0/0.56)	0.44 (75.0/0.69)	2.00 (75.0/0.58)	0.81 (37.5/0.26)
CSP-PSC	0.62 (87.5/0.87)	1.01 (75.0/0.85)	0.49 (87.5/0.98)	1.01 (62.5/0.36)	1.00 (87.5/0.98)
CSP-VRC	1.06 (50.0/0.28)	1.51 (37.5/0.25)	0.75 (62.5/0.60)	1.00 (75.0/0.33)	1.18 (12.5/0.26)
MCF-PSC	0.78 (37.5/0.17)	1.01 (62.5/0.58)	0.78 (75.0/0.68)	1.00 (50.0/0.58)	1.06 (25.0/0.27)
MCF-VRC	1.03 (75.0/0.62)	1.01 (62.5/0.76)	0.75 (25.0/0.18)	1.00 (50.0/0.33)	1.03 (50.0/0.68)
PSC-VRC	1.12 (50.0/0.42)	1.00 (75.0/0.82)	0.74 (25.0/0.13)	0.75 (87.5/0.73)	1.50 (25.0/0.29)

RESULTS

Eight participating laboratories reported approximately 1,450 MIC and IC_{50} readings from six single drugs and 15 two-drug combinations tested against five *Candida* isolates. MIC values of single drugs for the QC strain and for the four clinical strains are summarized in Table 1. As expected, all participating laboratories reproduced the QC range of *C. parapsilosis* ATCC 22019 and the previously reported high azole and echinocandin MICs for the four clinical *Candida* isolates (39, 40).

IC_{50} readings from the six drugs in 15 combinations, in 2-fold checkerboard dilutions, provided characteristic isobolograms for the calculation of FICs and ΣFICs. The visual and the spectrophotometric IC_{50}s were equally useful for the calculations of ΣFICs. Detailed median ΣFIC values calculated for five test strains based upon visual IC_{50} scores from eight laboratories are summarized in Table 2. Notably, median ΣFIC values were in the range of 0.51 to 1.12 (percent agreement, 25.0 to 87.5%; correlation coefficient, 0.17 to 0.96) for 15 drug combinations tested against *C. parapsilosis* (ATCC 22019). Median ΣFIC values were in the range of 0.51 to 1.31 (percent agreement, 37.5 to 87.5%; correlation coefficient, 0.18 to 0.91) for the same drug combinations tested against *C. albicans* (20533.043). Median ΣFIC values were in the range of 0.49 to 0.80 (percent agreement, 25.0 to 87.5%; correlation coefficient, 0.13 to 0.97) for 15 drug combinations tested against *C. albicans* (20464.007). Median ΣFIC values were in the range of 0.46 to 2.0 (percent agreement, 37.5 to 87.5%; correlation coefficient, 0.31 to 0.91) for 15 drug combinations tested against *C. glabrata* (20205.075). Median ΣFIC values were in the range of 0.38 to 1.50 (percent agreement, 12.5 to 75.0%; correlation coefficient, 0.26 to 0.98) for 15 drug combinations tested against *C. parapsilosis* (20580.070).

Median ΣFIC values calculated for the five test strains based upon spectrophotometric readings of the IC_{50}s from seven laboratories are summarized in Table 3; one participating laboratory did not provide this information. Notably, median ΣFIC values were in the range of 0.53 to 1.37 (percent agreement, 14.3 to 100%; correlation coefficient, 0.16 to 0.99) for 15 drug combinations tested against *C. parapsilosis* (ATCC

TABLE 3. Median ΣFICs of antifungal drug combinations for five *Candida* strains calculated from spectrophotometric readings reported by seven participating laboratories

Antifungal drug combination	Median ΣFIC (% agreement/correlation coefficient)				
	C. parapsilosis (ATCC 22019)	*C. albicans* 20533.043	*C. albicans* 20464.007	*C. glabrata* 20205.075	*C. parapsilosis* 20580.070
AMB-AND	0.53 (42.8/0.44)	0.81 (83.3/0.98)	0.46 (85.7/0.84)	1.04 (57.1/0.44)	0.20 (42.8/0.24)
AMB-CSP	0.56 (85.7/0.91)	0.90 (91.6/0.99)	0.56 (42.8/0.29)	1.06 (14.2/0.15)	0.43 (100/0.99)
AMB-MCF	0.42 (14.3/0.31)	1.06 (57.1/0.60)	0.67 (85.7/0.80)	1.06 (100/0.99)	0.40 (100/0.95)
AMB-PSC	1.04 (42.8/0.43)	0.59 (71.4/0.78)	0.56 (85.7/0.87)	0.35 (42.8/0.22)	0.59 (85.1/0.84)
AMB-VRC	1.01 (57.1/0.83)	0.51 (28.6/0.19)	0.59 (57.1/0.53)	0.38 (57.1/0.56)	1.04 (57.1/0.51)
AND-CSP	0.56 (100/0.99)	1.06 (57.1/0.67)	0.56 (42.8/0.49)	1.75 (100/0.99)	0.56 (14.2/0.24)
AND-MCF	0.56 (14.2/0.25)	1.75 (91.6/0.92)	0.56 (42.8/0.17)	1.75 (71.4/0.76)	0.65 (85.7/0.96)
AND-PSC	0.56 (100/0.99)	0.77 (71.4/0.76)	0.90 (85.7/0.97)	1.01 (72.8/0.57)	0.76 (85.7/0.87)
AND-VRC	1.06 (14.2/0.16)	0.76 (42.9/0.48)	0.77 (85.7/0.98)	0.76 (85.7/0.87)	1.01 (57.1/0.51)
CSP-MCF	0.62 (57.1/0.63)	1.31 (42.9/0.48)	0.55 (85.7/0.78)	2.00 (100/0.96)	0.56 (85.7/0.89)
CSP-PSC	0.56 (100/0.99)	1.02 (28.6/0.15)	0.68 (85.7/0.99)	1.01 (100/0.99)	0.68 (100/0.99)
CSP-VRC	1.06 (57.1/0.61)	1.02 (28.6/0.32)	0.76 (71.4/0.90)	1.01 (100/0.99)	1.04 (57.1/0.68)
MCF-PSC	1.01 (57.1/0.52)	1.02 (14.2/0.11)	0.76 (57.1/0.61)	1.01 (57.1/0.50)	1.03 (100/0.98)
MCF-VRC	0.76 (71.4/0.85)	1.02 (57.1/0.49)	0.75 (85.7/0.83)	1.01 (100/0.97)	1.03 (28.6/0.48)
PSC-VRC	1.37 (85.7/0.84)	0.52 (83.3/0.81)	0.76 (85.7/0.88)	0.76 (42.8/0.36)	1.50 (14.2/0.25)

TABLE 4. Synergistic interactions of antifungals assessed by ΣFICs derived from IC_{50}s obtained by visual readings

Candida strain	Antifungal combination	Visual median ΣFIC	No. of laboratories in agreement (%)
C. albicans 20464.007	AND-PSC	0.49	3 (37.5)
	CSP-MCF	0.44	4 (50.0)
	CSP-PSC	0.49	5 (62.5)
C. glabrata 20205.075	AMB-PSC	0.46	6 (75.0)
C. parapsilosis 20580.070	AMB-AND	0.38	7 (87.5)
	AMB-CSP	0.49	5 (62.5)
	AMB-PSC	0.50	3 (37.5)
	AND-PSC	0.50	6 (75.0)

TABLE 5. Synergistic interactions of antifungals assessed by ΣFICs derived from IC_{50}s obtained by spectrophotometric readings

Candida strain	Antifungal combination	Spectrophotometric median ΣFIC	No. of laboratories in agreement (%)
C. parapsilosis ATCC 22019	AMB-MCF	0.42	5 (71.4)
C. albicans 20464.007	AMB-AND	0.46	4 (57.1)
C. glabrata 20205.075	AMB-PSC	0.35	6 (85.7)
	AMB-VRC	0.38	5 (71.4)
C. parapsilosis 20580.070	AMB-AND	0.20	7 (100)
	AMB-CSP	0.43	5 (71.4)
	AMB-MCF	0.40	6 (85.7)

22019). Median ΣFIC values were in the range of 0.52 to 1.75 (percent agreement, 28.6 to 91.6%; correlation coefficient, 0.11 to 0.9) for 15 drug combinations tested against *C. albicans* (20533.043). Median ΣFIC values were in the range of 0.46 to 0.90 (percent agreement, 42.8 to 85.7%; correlation coefficient, 0.17 to 0.99) for 15 drug combinations tested against *C. albicans* (20464.007). Median ΣFIC values were in the range of 0.35 to 2.00 (percent agreement, 14.2 to 100%; correlation coefficient, 0.15 to 0.99) for 15 drug combinations tested against *C. glabrata* (20205.075). Median ΣFIC values were in the range of 0.20 to 1.50 (percent agreement, 14.2 to 100%; correlation coefficient, 0.24 to 0.99) for 15 drug combinations tested against *C. parapsilosis* (20580.070).

Overall, the IC_{50}s for all antifungal combinations were either lower than or equal to the MICs of the corresponding single drugs against all isolates tested. A majority of the ΣFIC values suggested indifference (0.51 to 2.0), but importantly, no antagonistic interactions (≥4.0) were observed. Eight synergistic interactions (ΣFIC ≤ 0.5) among two-drug combinations of AMB, AND, CSP, MCF, and PSC were evident from the median ΣFIC values derived from the visual scoring of the IC_{50}s for tests involving four out of the five *Candida* species investigated (Table 4). AND-PSC synergism was seen against at least two *Candida* isolates. The percent agreements were 37.5 to 87.5% for the synergistic interactions. Seven synergistic interactions (ΣFIC ≤ 0.5) between drug pairs among AMB, AND, CSP, MCF, PSC, and VRC were suggested by median ΣFIC values derived from spectrophotometric IC_{50}s for tests involving three out of the five *Candida* species investigated (Table 5). AMB-AND and AMB-MCF synergisms were observed against at least two *Candida* isolates. The percent agreements were 37.5 to 87.5% for synergistic interactions. Overall, both methods were in agreement for AMB-PSC synergistic interactions for *C. glabrata* 20205.075 and for AMB-AND synergistic interactions for *C. parapsilosis* 20580.070, with 75 to 100% agreements. Notably, the most common drug in the synergistic combination was AMB.

DISCUSSION

The observations obtained in this collaborative study are useful for advancing the long-term goal of developing a standard method for antifungal combination testing in clinical laboratories. We have extended our preliminary report on multilaboratory antifungal combination testing with a QC *Candida krusei* strain by the testing of more drug combinations with an expanded panel of yeast strains (12). Notably, a number of clinical *Candida* isolates with known resistance to azoles or echinocandins were tested and found to be susceptible to two drug combinations. None of the 15 drug combinations tested showed any antagonism (ΣFIC ≥ 4). Our expanded use of resistant clinical isolates suggested that potentially any *Candida* strain, including strains resistant to one or more drugs, could be tested reproducibly for *in vitro* susceptibility to various drug combinations. However, relatively high percent disagreements among participants were observed for many drug combinations tested with various *Candida* strains. Although few synergistic interactions were observed by visual or spectrophotometric endpoint readings, the ΣFIC values calculated from two methods were congruent in only two instances. Clearly, much more work lies ahead before a reproducible and practicable method can be identified for routine use in clinical laboratories.

This multilaboratory study expands upon reports from a number of laboratories suggesting that the CLSI M27-A3 method can be adapted to obtain reproducible results for combinations of antifungal drugs against yeasts (11, 25, 28, 38). For example, Barchiesi et al. previously reported that the combination of AMB and CSP produced enhanced activity against *C. parapsilosis* (9), which is similar to the observations of our study. However, Nishi et al. reported that MCF and VRC displayed synergistic activity against *C. glabrata* (34), but in the present study this combination was indifferent, possibly due to the different strains tested. Additionally, the distribution of median ΣFIC values in this study was skewed, and percent disagreements were low, with very few significant correlation coefficients. We believe that an inconsistent endpoint recording on the 0-to-4 scales in various laboratories was not the reason for the skewed distribution derived from IC_{50}s, since the visual image guide provided to each laboratory before the start of the study previously yielded impressive interlaboratory percent agreements (12). We find it hard to explain why there is so much disagreement observed for spectrophotometric readings, since it does not suffer from an individual operator's bias in the recording of endpoints. A contributing element for disagreements in this study could be that drugs with either fungistatic or fungicidal modes of actions were tested. Previous

reports of the testing of antibacterial combinations indicated that FIC predictions based upon checkerboards were more reliable for bactericidal activities rather than bacteriostatic activities (31, 45). In this context, it is noteworthy that maximal synergy combinations ($\Sigma FIC \leq 0.5$) were seen with amphotericin B, which has a fungicidal mode of action. The use of more stringent endpoints is clearly warranted. The utility of using MIC_{75}s or even MIC_{90}s instead of IC_{50}s was suggested previously in an exquisite comparison of different drug interaction models by Meletiadis et al. (32). Therefore, it would be worthwhile to investigate if the use of the IC_{90} or IC_{100} would improve interlaboratory reproducibility in future studies.

The format of the checkerboard dilution method for antimicrobial combination testing is familiar to clinical laboratories that already use CLSI broth microdilution methods for antifungal testing (13). There have been previous reports to indicate that the laboratory evidence of synergy between antifungal combinations based upon ΣFICs derived from checkerboard dilutions could be successfully correlated with clinical evidence of optimal outcomes in recalcitrant yeast infections (4, 21, 47). Earlier reports of antibacterial combination testing also indicated that FICs derived from checkerboard dilutions provide good predictions of bactericidal activity (31, 45). At the same time, ΣFIC values derived from checkerboard dilutions also present a number of problems (15, 24, 32, 36). These problems include controversial results that can be obtained based on the criteria used to evaluate antifungal interactions, such as endpoint determinations, the reading method, and analyses of results (15). In addition to methodological problems, the calculation of the ΣFIC assumes that all antifungal drugs interact with each other in a linear model (one dimensional), providing an all-or-none view, thus artificially creating ΣFIC values (15, 24, 36). An alternative in the form of response surface modeling was proposed to accommodate various dose concentrations of test drugs; applications of this model to antifungal combinations have yielded mixed results (22, 32, 49).

Other methods such as time-kill assays and Etest have been developed to overcome the suggested limitations of checkerboard dilutions and ΣFIC determinations (11, 25, 29, 44). The time-kill method is considered superior because of the time course activity of the two drugs in combination (11, 29). However, the time-kill method might be too labor-intensive and time-consuming for busy clinical laboratories; apart from these difficulties, it requires a fixed inoculum, fewer drug concentrations, and readings at one time point (25, 29). The Etest is another method used to perform antifungal combination testing in clinical laboratories (29). This method is simple to perform and time efficient. However, different classes of antifungal drugs show variable diffusion patterns, resulting in dissimilar ellipse patterns and difficult endpoint readings (29). Thus, it is more likely that an eventual method for antifungal combination testing in clinical laboratories would represent a compromise that balances ease of use, accuracy, reproducibility, and predictability.

ACKNOWLEDGMENTS

We thank Adriana Verschoor for valuable editorial comments.

This study was supported in parts with funds from the Wadsworth Center Clinical Laboratory Reference System (V.C.). Additional support came in the form of investigative grants from Astellas USA, Merck & Co., Pfizer Inc., and Schering-Plough Research Institute (V.C.). Trek Diagnostic Systems partially subsidized the cost of 96-well custom plates.

The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention.

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