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In Vitro Susceptibilities of Sucrose-Negative *Candida tropicalis*, *Candida lusitaniae*, and *Candida norvegensis* to Amphotericin B, 5-Fluorocytosine, Miconazole, and Ketoconazole

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The MICs and minimal lethal concentrations of four antimycotics, amphotericin B, 5-fluorocytosine, miconazole nitrate, and ketoconazole, were determined for 25 yeast isolates representing species uncommonly implicated in candidiasis. A microdilution procedure was employed with complex and synthetic media. The isolates, in general, were susceptible to the same antimicrobial agents shown to be effective against *Candida albicans*, but differences between some of the species in relative susceptibilities to the antifungal agents were noted. Isolates of atypical sucrose-negative *Candida tropicalis* were similar in their susceptibility patterns to typical isolates of the species. Relative resistance to amphotericin B, miconazole nitrate, and ketoconazole was noted for two *Candida lusitaniae* isolates, but all strains were susceptible to 5-fluorocytosine. *Candida norvegensis* isolates were more resistant to miconazole and ketoconazole than *C. albicans* clinical isolates. The microtiter system was satisfactory for determining minimal inhibitory concentrations, but the system is not recommended for detecting finite differences in drug susceptibilities or for detecting drug synergism.

Most studies on the in vitro antimycotic susceptibilities of clinical yeasts have been performed with *Candida albicans* or typical *Candida tropicalis* isolates (7, 16). Ahearn et al. (1) described a sucrose-negative (sn) variant of *C. tropicalis* from three cases of systemic candidiasis. Subsequently, this variant and a phenotypically similar yeast, *Candida lusitaniae*, have been reported in clinical specimens from diverse sites in the United States (2, 6, 10). Little or no information is available on the drug susceptibilities of these yeasts. The purpose of this investigation is twofold: (i) to determine drug susceptibilities of some of the less common etiological agents of candidiasis and (ii) to compare the drug susceptibilities determined by a microtiter technique with established values for standard strains.

MATERIALS AND METHODS

Cultures and inocula. Thirty yeast cultures, including two control *C. albicans* cultures and three control *C. tropicalis* isolates, were examined (Table 1). All cultures were maintained on Sabouraud glucose agar slants (SAB; Difco Laboratories, Detroit, Mich.). Inocula for the drug susceptibility studies were prepared with cells grown for 24 h on SAB at room temperature (18 to 24°C). Cells from the 24-h cultures were suspended in the various assay media to a turbidity equivalent to that of a 0.5 MacFarland standard (a range of 5×10^5 to 2×10^6 CFU/ml for the strains studied).

Assay media. The assay media were: yeast nitrogen base (YNB; Difco), a basic salts solution containing trace elements and vitamins with ammonium sulfate as the nitrogen source and supplemented with 4.0% glucose adjusted with 1 N NaOH to pH 7.0; antibiotic 20 medium (M-20; Difco) containing 1.5 g of beef extract, 6.5 g of yeast extract, 5.0 g of peptone, 10.0 g of tryptone, 11.0 g of glucose, 3.5 g of NaCl, 3.68 g of K_2HPO_4, and 1.32 g of KH_2PO_4 per liter of deionized water (pH 6.6); and synthetic amino acid medium for fungi (SAAMF; GIBCO Laboratories, Grand Island, N.Y.). SAAMF is a mixture of 16 amino acids, B vitamins, basic salts, and 2% glucose adjusted to pH 7.4 with morpholinepropanesulfonic acid and Tris buffers (3). The SAAMF and YNB media were sterilized by filtration, whereas M-20 was autoclaved. The defined media were prepared in concentrated strength (YNB [10×] and SAAMF [2×]).

Antimicrobial agents. Amphotericin B (AMB) (Fungizone; E. R. Squibb & Sons, Princeton, N.J.; control 8C734) was diluted according to the instructions of the manufacturer with 5 ml of sterile deionized water which was injected directly into the vial to yield a 1.0% stock solution. A 0.5% stock solution of 5-fluorocytosine (5-FC; Hoffmann-La Roche Inc., Nutley, N.J.; lot 060053) was prepared in sterile deionized water. Miconazole nitrate (MCZ; Johnson & Johnson Pharmaceutical Research, New Brunswick, N.J.; control 45003B, lot B74/1) was dissolved (5 mg/ml) in dimethyl sulfoxide. A 5% solution of ketoconazole (KTZ; Janssen Pharmaceutia, New Brunswick, N.J.) was prepared in 0.4 N HCl. Rifampin (CIBA Pharmaceutical Co., Summit, N.J.; lot M-1227) was dissolved in dimethyl sulfoxide to yield a 1.0% solution. The vial of stock AMB was wrapped in aluminum foil and frozen at −15°C. All other antimicrobial agents were refrigerated until used.

MIC. A microtiter assay system with sterile trays with 96 flat-bottomed wells (Linbro Scientific, Inc., Hamden, Conn.) and multichanneled variable volume micropipettors (Flow Laboratories, Inc., Rockville, Md.) was employed. The stock solutions of antimicrobial agents were appropriately diluted in the various assay media to give the following working solutions: AMB, 400 μg/ml; 5-FC, 400 μg/ml; MCZ, 1,600 μg/ml; and KTZ, 1,600 μg/ml. The assay broths were dispensed (25 μl) into all wells of separate trays. An equal volume of the drug solution was added to the first well of each row of the microtiter trays, and the contents of the well were mixed thoroughly with an automatic pipettor. After mixing, 25 μl was transferred from the first well and mixed as described above with the contents of the second well. Repetition of this process for successive wells yielded the appropriate serial dilutions. The last two wells were left drug free to serve as controls. The first 11 of the 12 wells of each

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TABLE 1. Yeast strains tested in study

Isolate code	Species	Georgia State University culture no.	Source
1	*C. tropicalis* (sn)	80-005	Spleen
2	*C. tropicalis* (sn)	80-004	Liver
3	*C. tropicalis* (sn)	80-003	Blood
4	*C. tropicalis* (sn)	78-012	Blood
5	*C. tropicalis* (sn)	78-013	Blood
6	*C. tropicalis* (sn)	78-014	Blood
7	*C. tropicalis* (sn)	78-050	Blood
8	*C. tropicalis* (sn)	78-016	Blood
9	*C. tropicalis* (sn)	78-019	Blood
10	*C. tropicalis* (sn)	78-020	Blood
11	*C. tropicalis* (sn)	78-021	Blood
12	*C. tropicalis* (sn)	78-022	Blood
13	*C. tropicalis* (sn)	78-039	Blood
14	*C. tropicalis* (sn)	78-049	Blood
15	*C. tropicalis* (sn)	79-001	Sputum
16	*C. tropicalis* (sn)	80-001	Sputum
17	*C. lusitaniae*	78-028	Sputum
18	*C. lusitaniae*	78-060	Blood
19	*C. lusitaniae*	78-061	Blood
20	*C. lusitaniae*	78-062	Lung
21	*C. lusitaniae*	78-063	Lung
22	*C. lusitaniae*	78-064	Unknown
23	*C. lusitaniae*	79-003	Sputum
24	*C. lusitaniae*	80-014	Tongue
25	*C. tropicalis*[a]	223	Bronchial washings
26	*C. tropicalis*[a]	224	Maxillary sinus
27	*C. tropicalis*[a]	CDC-38	Unknown
28	*C. norvegensis*	226	Urine
29	*C. norvegensis*	232	Urine
30	*C. albicans*[a]	Ca9	Feces
31	*C. albicans*[a]	Ca30	Vaginal secretions

[a] Control organisms.

row were inoculated with 25 μl of a standardized cell suspension. To all wells, 150 μl of assay broth was added, yielding a final antimicrobial concentration range of 100 to 0.18 μg/ml for KTZ and MCZ and 25 to 0.045 μg/ml for 5-FC and AMB. The final volume of each well was 0.2 ml. *C. albicans* strains Ca9 and Ca30 from clinical sources with MICs and minimal lethal concentrations (MLCs) established by tube dilution procedures were used as controls (8). One control strain and seven study strains were tested in each tray.

The trays were incubated in the dark at room temperature and examined at 24, 48, and 72 h. The MIC was defined as the lowest concentration at which there was no visible turbidity. The MICs were recorded at 48 h. The reported MICs were obtained in two to three repeated tests.

MICs to AMB in M-20 and MICs to 5-FC, MCZ, and KTZ in YNB medium were determined for all yeasts in the study. Twenty-two representative isolates were further tested against all antifungals in SAAMF for comparison of results in different media.

MLC. After 72 h, select wells showing no growth were mixed thoroughly and subcultured to determine fungistatic or fungicidal activity. The well containing the highest concentration of antimicrobial agents and no growth and, from this point, every other well up to the first well with growth were subcultured (0.1 ml) with an automatic pipettor into tubes containing 10.0 ml of yeast extract broth (10.0 g of yeast extract, 5.0 g of glucose, 5.0 g of peptone per liter of deionized water). These cultures were incubated for 24 to 48 h at room temperature on a rotating drum. The MLC was recorded as the lowest concentration of drug that did not yield growth.

Synergistic activity by microtiter assay. Possible synergy between KTZ and rifampin was examined with a constant subinhibitory concentration of rifampin (10 μg/ml) and with increasing concentrations of KTZ. A KTZ working solution of 100 μg/ml was serially diluted in SAAMF as described above. After the wells were inoculated, 50 μl of SAAMF was added to increase the volume to 0.1 ml per well. Finally, 0.1 ml of SAAMF containing rifampin (20 μg/ml) was added for a final volume of 0.2 ml per well. The concentration of KTZ theoretically ranged from 6.25 to 0.01125 μg/ml. All tests were performed in duplicate and incubated in the dark at room temperature for 72 h. Synergy was designated as a twofold decrease in MICs when the two drugs were combined as compared with either drug alone.

RESULTS

The relative susceptibilities of the yeasts to AMB, 5-FC, MCZ, and KTZ are given in Table 2. For the most part, the fungicidal antimicrobial AMB gave MLCs only slightly higher than the MICs. Only the seven *C. lusitaniae* isolates had MLCs to AMB that were more than two dilutions greater than their MICs. In contrast, MLCs for the static antimicrobial agents, 5-FC, MCZ, and KTZ, exceeded the MICs by at least two dilutions for most yeasts. The *C. tropicalis* (sn) strains appeared more susceptible to AMB than to the other antimicrobial agents (Fig. 1). The MIC with AMB was generally well defined, and development of resistant colonies was not observed. MICs of KTZ and MCZ were less definite, particularly if the plates were examined after 48 h, since there was more of a gradual decrease in growth with increasing antimicrobial agents rather than a marked transition. Resistance to 5-FC was noted for three isolates. These three isolates demonstrated inconsistent values of 6.3 to >25 μg/ml to inhibit growth in repeated tests. The three control *C. tropicalis* strains demonstrated similar reactions to the

TABLE 2. Antimicrobial susceptibilities of yeasts[a]

Species	No. of isolates	AMB		5-FC		MCZ		KTZ	
		MIC	MLC	MIC	MLC	MIC	MLC	MIC	MLC
C. albicans[b]	2	0.09–0.18[c]	0.78–1.6	0.09–1.6	0.39–>25	1.6–6.3	6.3–100	6.3	6.3–100
C. tropicalis[b]	3	0.39–0.78	0.39–1.6	0.05–0.39	0.18–>25	3.1–12.5	25–>100	6.3–12.5	50–100
C. tropicalis (sn)	16	0.18–0.39	0.78–3.1	0.15–>25	1.6–>25	3.1–6.3	6.3–>100	6.3–25	12.5–100
C. lusitaniae	7	0.39–6.3	1.6–>25	0.05–0.18	0.18–1.6	0.18–6.3	0.39–>100	0.18–6.3	0.78–12.5
C. norvegensis	2	0.39	3.1–6.3	25	>25	0.78	1.6–>50	0.78	25–>100

[a] AMB was assayed in M-20; 5-FC, MCZ, and KTZ were assayed in YNB.
[b] Control isolates.
[c] Range of MICs and MLCs in μg/ml.

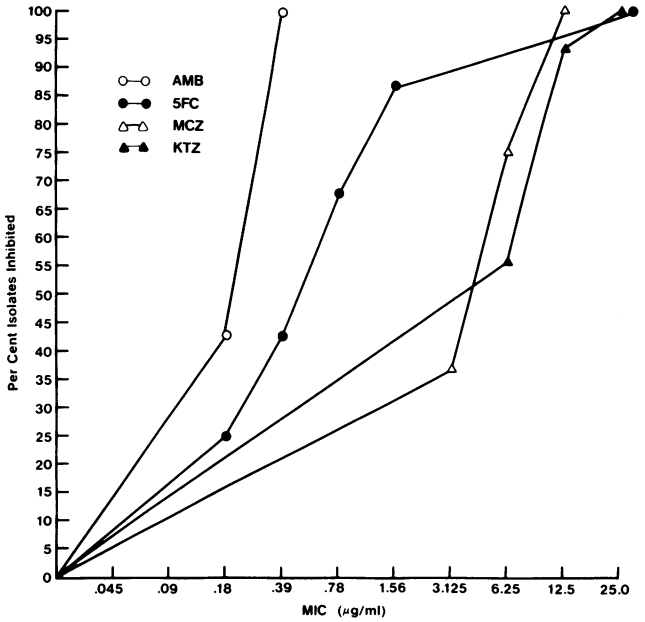

FIG. 1. MICs of 5-FC, MCZ, and KTZ for 16 *C. tropicalis* (sn) isolates.

antimicrobial agents; however, there was only survival rather than active growth in the higher concentrations of 5-FC. In contrast to results with *C. tropicalis* (sn), all *C. lusitaniae* isolates were quite susceptible to 5-FC and MCZ with sharp endpoints, and only one strain had an MIC for KTZ above 1.6 μg/ml (Table 2; Fig. 2). Two isolates (numbers 19 and 20) had relatively high MLCs to AMB in M-20. The *C. norvegensis* isolates were relatively resistant to 5-FC but susceptible to the imidazoles.

The effect of assay media on MICs of representative isolates is shown in Fig. 3. Most MICs to AMB for the 22 selected yeasts were identical or within one tube dilution in M-20 and SAAMF. The two *C. lusitaniae* strains (numbers 19 and 20) which appeared resistant to AMB in M-20 appeared susceptible to AMB in SAAMF. In SAAMF, all yeasts had MICs for 5-FC slightly higher than those found in YNB. The MICs of 5-FC were low enough to categorize most yeasts as susceptible, that is, <16 μg/ml (4). A few strains, notably *C. tropicalis* (sn) (numbers 8 and 9) and *C. norvegensis* (numbers 28 and 29), produced growth in assay wells at concentrations of 5-FC above 16 μg/ml. Similar resistance patterns were observed for most of these isolates in YNB.

Except for *C. norvegensis*, MICs for MCZ and KTZ determined in YNB frequently exceeded achievable serum levels, whereas isolates tested in SAAMF appeared extremely susceptible to MCZ and KTZ. The MICs of ≤0.18 μg/ml found in SAAMF for MCZ are in agreement with reported values for this medium (3).

No evidence of synergistic activity between KTZ and rifampin for *C. norvegensis* was obtained with the microtiter assay. This species was chosen for synergy study since it included the only isolates somewhat resistant to KTZ in SAAMF. The control strain of *C. albicans* (Ca30) also gave no evidence of a synergistic effect of the two drugs. However, the MIC of KTZ for the control strain was reduced by one dilution in the presence of rifampin as determined by a standard tube dilution procedure. Lack of synergistic inhibition of *Candida* spp. with combinations of rifampin and KTZ has been reported previously (7).

DISCUSSION

The *C. tropicalis* (sn) were similar in their in vitro susceptibilities (with selected antifungals) to typical isolates of the species and to values reported for *C. albicans* (11). Resistance to 5-FC is common among candidas, and it was observed for several of the *C. tropicalis* (sn) isolates. MICs to MCZ and KTZ in YNB indicated in vitro resistance to the imidazoles among both typical and atypical *C. tropicalis*. These data were in accord with MICs reported by Moody et al. (7) for *C. tropicalis* isolates tested by an agar dilution procedure.

In contrast to results obtained with YNB and SAB, the MICs to MCZ and KTZ for all yeasts determined in SAAMF were within achievable serum levels. Hoeprich and Huston (5) reported similar findings with SAAMF and suggested that MCZ and possibly other imidazoles are blocked from full expression of activity in more complex media but not in synthetic media.

Van den Bossche et al. (15) reported that the effects of MCZ appear pronounced in logarithmically growing cells, which suggests high metabolic activity enhances susceptibility to the drug. In this regard, the SAB used by Moody et al. (7) probably contained sterols or sterol precursors that antagonized the activity of the imidazoles and produced high MICs. As contrasted with SAAMF, we observed relatively high MICs with the imidazoles in YNB medium, although this defined medium is supposedly also free of sterols. YNB and SAAMF supported equivalent growth of most yeasts, but the MICs for MCZ and KTZ at 48 h were higher for most yeasts in YNB as compared with SAAMF; however, 24-h data appeared similar.

The two *C. lusitaniae* strains (numbers 19 and 20) were the only strains which gave MICs to AMB that exceeded attain-

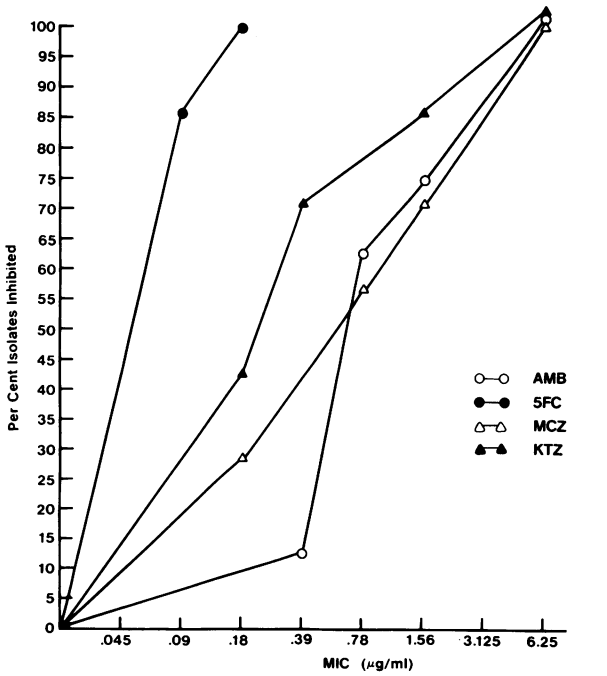

FIG. 2. MICs of AMB, 5-FC, MCZ, and KTZ for seven *C. lusitaniae* isolates.

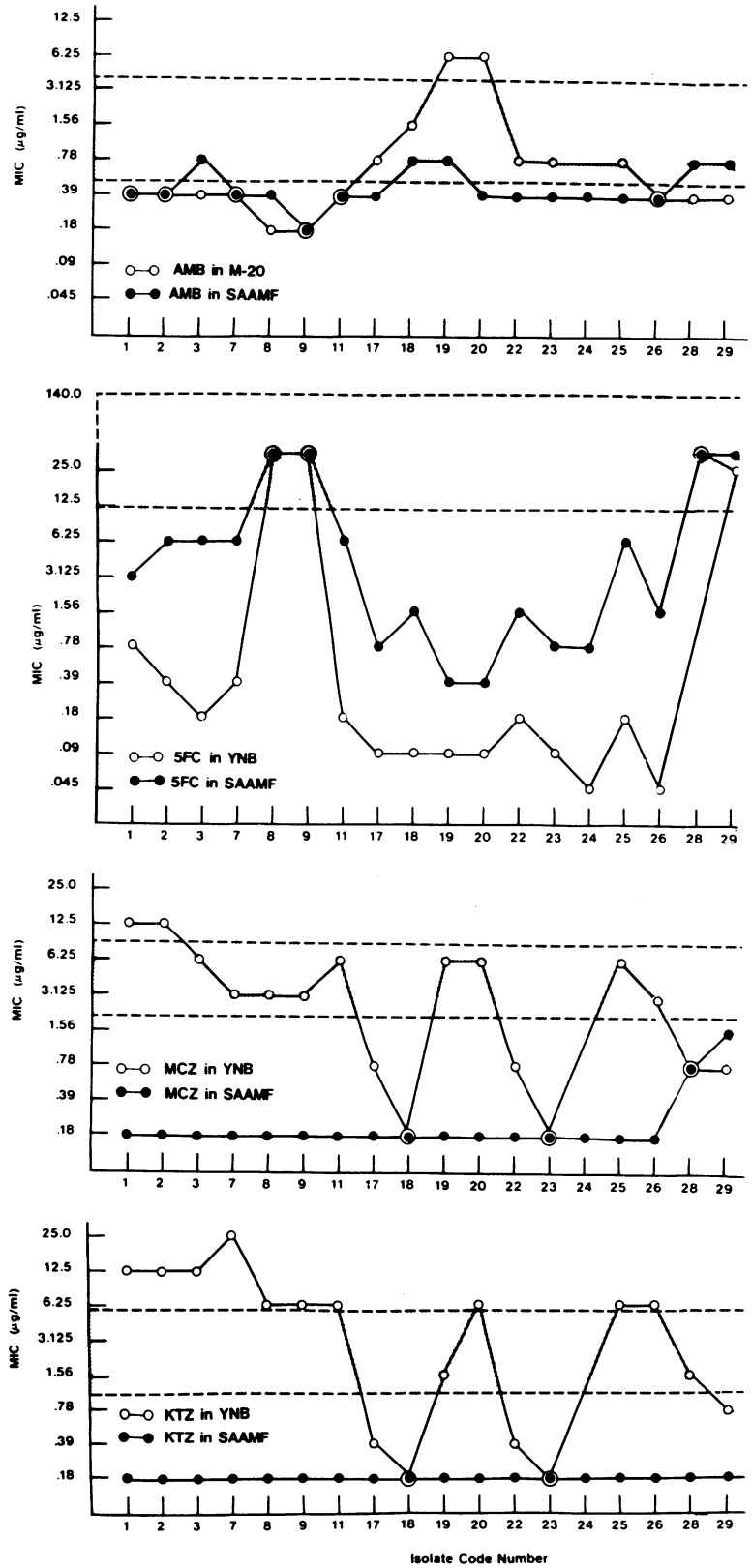

FIG. 3. MICs of AMB, 5-FC, MCZ, and KTZ in two different media, YNB and SAAMF, for selected yeasts. Concentration ranges within the dashed lines represent reported serum levels.

able serum levels. These yeasts were isolated from a leukemic patient receiving cytotoxic agents possibly responsible for mutagenic development of the polyene resistance; however, other *C. lusitaniae* isolates from the same patient were susceptible to AMB (6, 9). Both resistant isolates were previously reported to have MICs of 30 μg/ml for AMB at 24 h by a YNB agar dilution procedure (9). In our study, the two isolates were relatively resistant compared with other isolates examined on M-20 (MICs of 6.3 μg/ml). As noted above, YNB and SAAMF are synthetic media without sterols and other complex macromolecules that could interfere with AMB activity. Therefore, the resistant values for these strains in YNB reported by Pappagianis et al. (9) argue against sterol antagonism as an explanation for differences in MICs in M-20 and SAAMF for these particular isolates. The results seem to indicate a uniqueness of these strains, since the concentrations of sterols in M-20 were apparently insufficient to antagonize the AMB activity with all other isolates whose MICs in SAAMF were essentially the same as their MICs in M-20. In support of this supposition is the observation that the same two *C. lusitaniae* isolates were relatively resistant to MCZ and KTZ in YNB compared with the other isolates of this species. The relation of the unique results with these strains to a membrane phenomenon may be supported by the findings that inhibition by imidazoles has been related to an effect on steroid metabolism (14, 15). In other studies, the effects of KTZ on *C. albicans* have included selective inhibition of the mycelial phase (13) and inhibition of the respiration of logarithmic phase cells (12). The inhibitory effects of the imidazoles on yeasts appear to be strongly affected by conditions for their growth.

Since the development of in vivo polyene and imidazole resistance among some *C. lusitaniae* strains is possible, 5-FC may be the drug of choice for therapy. The MICs to 5-FC for all *C. lusitaniae* isolates were very low, and their MLCs were all ≤1.6 μg/ml. Apparently, 5-FC has a fungicidal effect with this species rather than a fungistatic effect. Furthermore, no evidence of resistance to 5-FC was noted in the assay tests.

Among those clinical isolates resistant to 5-FC were the two *C. norvegensis* isolates. This species is only occasionally isolated from clinical specimens, and it has not been established as an agent of adventitious infections. Unlike all other isolates, the susceptibilities to imidazoles for both *C. norvegensis* isolates were essentially the same in both YNB and SAAMF.

SAAMF appeared to provide optimal activity for most antifungals. Hoeprich and Huston (5) recommended SAAMF because of its lack of antagonism to antifungals, its nutritional adequacy for a wide variety of fungi, the superior stability of pH, and the certifiable absence of macromolecules, chelators, purines, pyrimidines, and sterols. However, they did observe that YNB appears to be optimal for testing 5-FC. This observation was supported by our results; MICs of 5-FC in YNB were generally lower than those seen with SAAMF.

The benefits of a microtiter system for screening antimicrobial susceptibilities of yeasts include savings in space, media, and time in handling large numbers of isolates. The actual incubation time, however, may need to be extended over that of a macro system for certain *Candida* strains. We found that the microtiter system provides relatively accurate MIC data but required somewhat more care in interpretation. We do not recommend our microtiter system for detecting finite differences in drug susceptibilities or for detecting drug synergism.

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