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In Vitro Studies of a New Antifungal Triazole, D0870, against *Candida albicans*, *Cryptococcus neoformans*, and Other Pathogenic Yeasts

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We investigated the effects of various assay conditions on the activity of D0870 against seven species of fungi in the broth macrodilution testing procedure proposed by the National Committee for Clinical Laboratory Standards (NCCLS). Multivariate analysis demonstrated that endpoint definition, starting inoculum size, medium composition, type of buffer, and length of incubation, but not pH or temperature, had significant effects on results. Increasing the inoculum from 10^2 to 10^5 yeast cells/ml raised the MICs for all isolates up to >75,000 fold. This effect was greatest when endpoints corresponded to a 90% reduction in visually determined turbidity (MIC_{90}), was less prominent with an 80% inhibition visual endpoint (MIC_{80}), and was nearly absent with a 50% endpoint measured by a spectrophotometer ($IC_{1/2}$). Differences due to medium composition were attributable to antibiotic medium 3 with RPMI and yeast nitrogen base media performing nearly identically. Under standardized conditions as specified in NCCLS document M27-P (*Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; Proposed Standard*, 1992), 79 strains (5 to 25 strains for each species) demonstrated median MIC_{80}s of 0.0037 and 0.0075 μg/ml for *Candida albicans* and *Cryptococcus neoformans*, respectively. In contrast, *Candida krusei* and *Torulopsis glabrata* had a median MIC_{80} of 1.0 μg/ml. Our studies indicate that the pathogenic yeasts *C. albicans* and *C. neoformans* are more susceptible to D0870 than other pathogenic yeasts.

D0870,(*R*)-2-(2,4-difluorophenyl)-1-(3-[(*E*)-4-(2,2,3,3-tetrafluoropropoxy)-styryl]-1H-1,2,4,-triazol-1-yl)-3-(1H-1,2,4-triazol-1-yl)propan-2-ol, is a new antifungal agent that is the enantiomer of ICI 195,739. The pharmaceutical sponsor (Zeneca Pharmaceutical Corporation, Macclesfield, England) and recent published reports have indicated that mice infected with either *Candida albicans* or *Cryptococcus neoformans* respond to oral administration of D0870 at doses approximately six times lower than would be used for fluconazole (6). Reflecting in vivo drug activity by in vitro susceptibility testing would be useful in preparation for clinical trials with this agent. To this end, the National Committee for Clinical Laboratory Standards (NCCLS) has recently proposed a broth macrodilution reference method for susceptibility testing of yeasts (document M27-P [22]). Although experience with this method has been reported for amphotericin B, flucytosine, ketoconazole, and fluconazole (7, 9), D0870 has not yet been evaluated. Thus, it is not yet known whether the conditions proposed by the NCCLS will discern differences in activity of D0870 among strains or species.

In this report, we have analyzed the effect of changing each of several test conditions that influence test results with other antifungal agents (2, 5, 10, 11, 18, 19, 29). Because of accumulating reports of clinical resistance of *Candida krusei* to treatment with another triazole, fluconazole (1, 4, 17, 20, 24, 28, 30), we have paid special attention to the ability of various test conditions to provide discrimination in results produced with this species in comparison to *C. albicans*. Our findings support the use of test conditions in keeping with NCCLS guidelines which successfully distinguish most strains of *C. albicans* and *C. neoformans* as susceptible relative to *C. krusei* and *Torulopsis glabrata*.

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MATERIALS AND METHODS

Drug. Micronized D0870 was provided by the pharmaceutical sponsor (Zeneca) and was stored desiccated at room temperature until use. A stock solution was prepared by dissolving D0870 in polyethylene glycol 400 (Aldrien Chemical Co. Inc., Milwaukee, Wis.) heated to 75°C in a water bath to a final concentration of 800 μg/ml, and the preparation was divided for storage at −70°C prior to use.

Yeasts. A group of seven yeast isolates (one each of *C. albicans*, *Candida lusitaniae*, *Candida tropicalis*, *Candida parapsilosis*, *T. glabrata*, *C. krusei*, and *C. neoformans*) was used for the initial studies. In addition, 72 other strains of yeasts were tested under a single set of standardized conditions. All told, there were 25 *C. albicans*, 9 *C. lusitaniae*, 10 *C. tropicalis*, 10 *C. parapsilosis*, 10 *T. glabrata*, 5 *C. krusei*, and 10 *C. neoformans* isolates. *C. krusei* isolates were acquired from the Medical College of Virginia, Richmond (four strains), and St. John's Hospital, Detroit, Mich. (one strain). Strains of other *Candida* species were genetically distinct blood isolates from the University of Iowa (25, 26). *C. neoformans* isolates were obtained from Virginia Medical College and have been studied previously (7, 9). Prior to use in these studies, isolates had been stored in yeast nitrogen broth (YNB; Difco Laboratories, Detroit, Mich.) with 10% glycerol at −70°C. To prepare fresh starting inocula, the strains were thawed and inoculated on Sabouraud dextrose

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TABLE 1. Influence of different test conditions on results produced by broth macrodilution susceptibility testing of D0870

Variable and condition	Geometric mean (μg/ml)	P value
Endpoint		
MIC_{100}	1.0261	<0.001
MIC_{90}	0.5404	
MIC_{80}	0.1314	
$IC_{1/2}$	0.0645	
Inoculum size		
1×10^2–5×10^2	0.1320	<0.001
1×10^3–5×10^3	0.2111	
1×10^4–5×10^4	0.7018	
1×10^5–5×10^5	4.9015	
pH		
7.4	0.1272	0.611
7.0	0.2918	
6.0	0.2639	
5.0	0.1937	
4.0	0.2951	
3.0	0.1569	
Medium		
RPMI 1640	0.2525	<0.03
YNB	0.2576	
AM3	0.4784	
Buffer		
MOPS	0.2933	<0.001
HEPES	0.1370	
Phosphate	0.1056	
Temp (°C)		
30	0.2809	0.447
35	0.2509	
37	0.1822	
Time		
1st reading	0.1951	<0.001
2nd reading	0.3514	

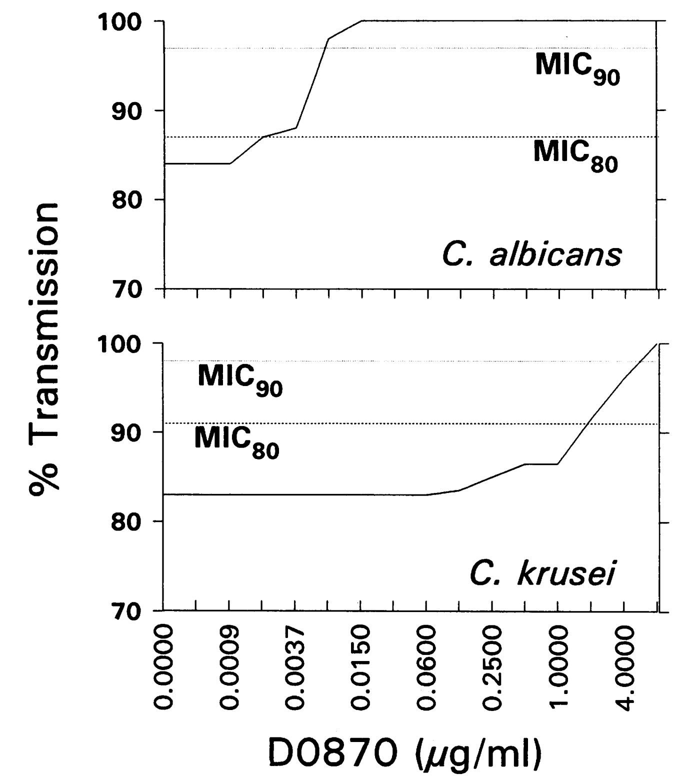

FIG. 1. Effect of increasing concentrations of D0870 on growth of *C. albicans* and *C. krusei*. Horizontal dotted lines indicate the turbidity of drug-free control growth diluted with medium in a ratio of 1 to 10 (MIC_{90}) or 1 to 5 (MIC_{80}) as labelled.

agar plates (Becton Dickinson Microbiology Systems, Cockeysville, Md.) repeatedly for overnight growth at 37°C.

Media and buffers. Used in most studies was RPMI-1640 (Sigma Chemical Co., St. Louis, Mo.), buffered with morpholinopropanesulfonic acid (MOPS; Sigma Chemical Co.) at a final concentration of 0.165 M to a pH of 7.0. Where specified in the results, YNB or antibiotic medium 3 (Difco) was substituted for RPMI-1640. In other studies, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; Sigma) or sodium phosphate was substituted for MOPS, and the pH was adjusted to between 3.0 and 8.0.

Susceptibility testing. Broth macrodilution susceptibility tests were performed in conformity with the NCCLS reference method [22] with conditions modified for specific experiments as described in the text. Briefly, twofold dilutions of the antifungal agents were prepared in medium to concentrations ranging from 0.0018 to 16.0 μg/ml. Yeast inocula were adjusted by spectrophotometer to 1×10^3 to 5×10^3 yeast cells per ml. Yeast inoculum (0.9 ml) and diluted drug (0.1 ml) were mixed (final volume, 1.0 ml) in polystyrene tubes and incubated at 35°C without agitation.

Several endpoints were recorded for each susceptibility test. As the standard endpoint, the MIC_{80} was defined as the lowest concentration that resulted in visual turbidity less than the turbidity of a drug-free control growth after a fivefold dilution (one part yeast suspension, four parts medium), was determined after 48 h of incubation (72 h for *C. neoformans*). The MIC_{90} was similarly defined in comparison to a 10-fold dilution of drug-free growth, and an MIC_{100} was defined as the lowest concentration with complete absence of visible turbidity. In addition, a turbidimetric endpoint ($IC_{1/2}$) was determined as the lowest drug concentration that resulted in the following: $\%T > \%T_{control} + [0.5(100 - \% T_{control})]$, where $\%T$ is the percent transmission and $\%T_{control}$ is the turbidity in the drug-free control tube (10).

One strain of *C. albicans* was included as a quality control strain with each experiment. The results reported are the product of at least two separate experiments that agree within a twofold range.

Statistical procedures. Significance of the effect of varying test conditions was tested by an analysis of variance procedure as implemented by Systat (Systat, Inc. Evanston, Ill.). The significance of differences between groups tested under standard conditions was determined by the Mann-Whitney U test.

RESULTS

Overall effects of changing test conditions. Results with one strain of each of seven species were analyzed to identify test conditions which influenced test results. Test parameters that had significant effects included endpoint definition, starting inoculum size, medium composition, different buffers, and length of incubation (Table 1). In contrast, little or no systematic effect was detected with changes in medium pH or temperature.

Effect of different endpoints. The relationship of MIC_{90} and MIC_{80} endpoints to resulting turbidity after incubation in

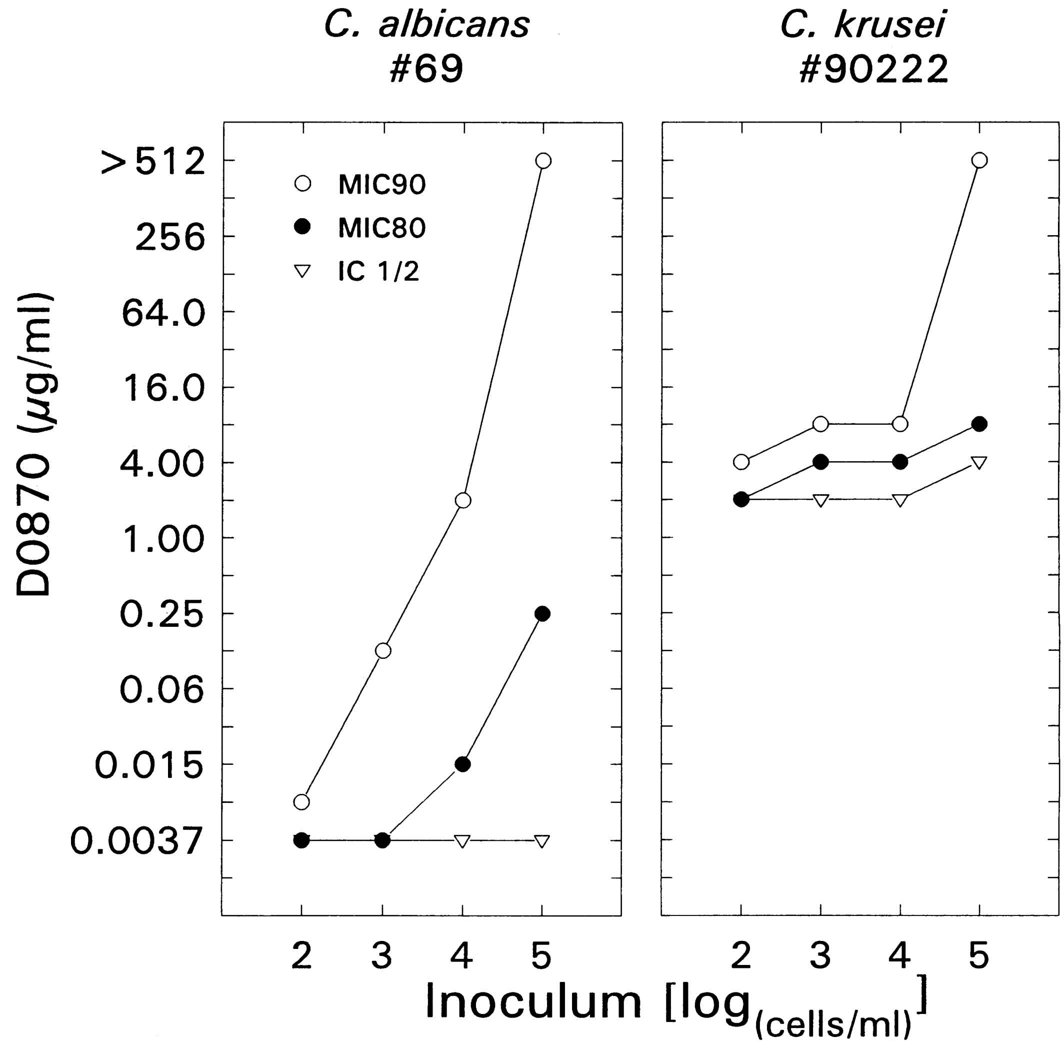

FIG. 2. Effect of varying the starting inoculum from 10^2 to 10^5 yeast cells per ml on different broth macrodilution endpoints for *C. albicans* and *C. krusei.*

different concentrations of D0870 is depicted in Fig. 1 for single strains of *C. albicans* and *C. krusei*. As can be seen, D0870 inhibits both strains but this effect occurs at a much lower concentration for the strain of *C. albicans*. The MIC_{80} endpoint detects this difference as 0.0018 and 2.0 μg/ml for *C. albicans* and *C. krusei*, respectively, as does the $IC_{1/2}$ endpoint (data not shown). On the other hand, at concentrations many fold higher than that which produces the predominant inhibitory effect, slight amounts of growth for this and many other strains continued to be evident. This behavior rendered the MIC_{100} insensitive to the predominant drug effect detected by the less stringent MIC_{80} and $IC_{1/2}$. It should also be noted that the MIC_{90} endpoint approaches the level of visual detection, and to the naked eye the low levels of growth at these concentrations may be indistinguishable from the MIC_{90}. As a result, interobserver variation in reading MIC_{90} endpoints would be expected to be more likely, obscuring differences between *C. albicans* and *C. krusei.*

Effect of other significant variables. When the starting yeast inoculum was varied from 10^2 to 10^5 cells per ml for each of seven yeasts, MIC results for D0870 increased (Fig. 2). For MIC_{90} results, differences were as much as >75,000 fold. Similar differences but of lesser magnitude were found with MIC_{80} endpoints as well. In contrast, $IC_{1/2}$ results were independent of an inoculum effect with all results for each strain within a twofold range.

When susceptibility testing was performed with different media, most of the differences were between results with antibiotic medium 3 as compared with either RPMI 1640 or YNB (Table 1). For some isolates, differences with MIC_{90} results were as much as 64 fold, and the strain of *C. albicans* was indistinguishable from that of *C. krusei*. With other isolates and other endpoints, the differences when antibiotic medium 3 was used were less pronounced.

Comparing results obtained on the first and second days of reading (that is, 24 or 48 h for all species except *C. neoformans*, which was read at 48 and 72 h of incubation), MIC_{90}s increased from 16- to 32-fold. As with other variables, MIC_{80} results were less effected by differences in incubation time, and differences between first- and second-day readings ranged only from 4- to 16-fold.

Factors with little or no influence. In studies that varied the pH or the incubation temperature, there was no overall

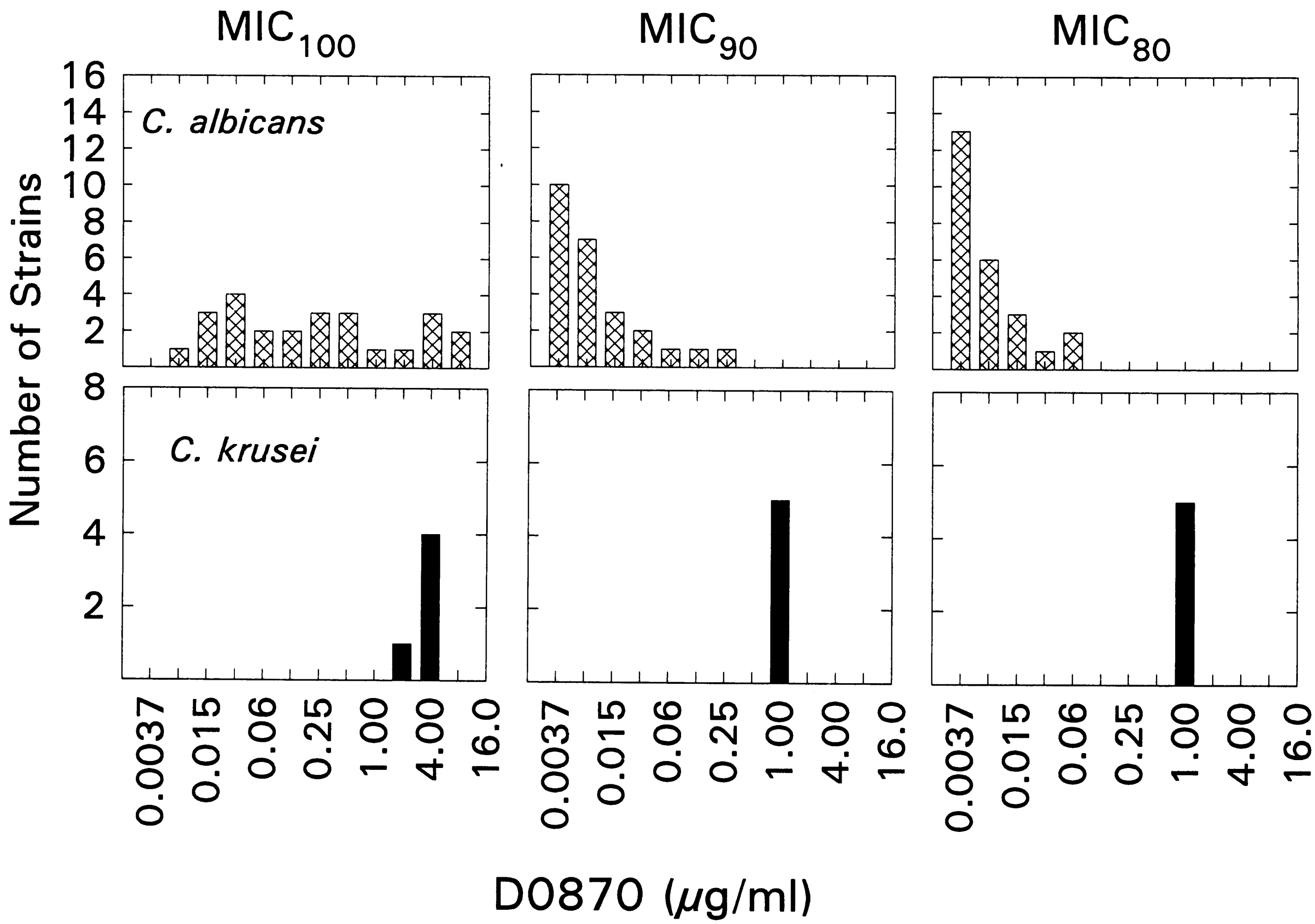

FIG. 3. Distributions of broth macrodilution results with different endpoints for 25 strains of *C. albicans* and 5 strains of *C. krusei*.

influence apparent with either variable. However, with an occasional isolate, a pronounced pH effect was noted. For example, with *C. lusitaniae*, MIC_{90}s were 0.06, 0.25, 0.25, and 0.5 μg/ml with pHs of 7.4, 6.0, 5.0, and 4.0, respectively.

Range and distribution of results under standard conditions. The above studies indicated that test conditions recommended for testing other drugs in the NCCLS reference procedure could be applied to testing of D0870 without modification. Accordingly, those test conditions were used to study 72 additional strains of yeasts. When the distribution of results for each species was analyzed by different endpoints, significant differences were found. For *C. albicans*, MIC_{80} results showed a median of 0.0037 μg/ml, with values for most strains tightly clustered around this value (Fig. 3). In contrast, with the more stringent endpoints (MIC_{90} and MIC_{100}), the median distribution shifted higher and the distribution broadened considerably. Results with *C. tropicalis*, *C. parapsilosis*, and *C. lusitaniae* produced similar patterns (data not shown). For *C. krusei*, results with MIC_{80} were much higher than were found with *C. albicans* but the MIC_{90} and the MIC_{100} results remained nearly identical to the MIC_{80} results. Consequently, only the MIC_{80} produced a wide separation between the two species. *T. glabrata* showed a pattern similar to that for *C. krusei*.

The median and range for each species tested with the MIC_{80} endpoint is shown in Table 2. *C. albicans* and *C. neoformans* are the most susceptible species, with 31 of the 35 strains inhibited by concentrations of 0.03 μg/ml or less. *C. tropicalis*, *C. parapsilosis*, and *C. lusitaniae* are slightly but significantly less susceptible species. *C. glabrata* and *C. krusei* have significantly higher MIC_{80}s than all other species, ranging from 0.125 to 4.0 μg/ml.

TABLE 2. Susceptibility results determined under standardized conditions[a]

Strain (*n*)	MIC_{80} (μg/ml)	
	Median	Range
C. albicans (25)	0.0037	0.0037–0.06
C. neoformans (10)	0.0075	0.0075–0.125
C. lusitaniae (9)	0.06	0.015–0.06
C. parapsilosis (10)	0.06	0.015–0.5
C. tropicalis (10)	0.06	0.03–2.0
T. glabrata (10)	1.0	0.125–4.0
C. krusei (5)	1.0	1.0

[a] Differences between species were tested by Mann-Whitney U tests, and $P < 0.05$ was taken as the critical level of significance. *C. albicans* and *C. neoformans* were more susceptible than all other species; *C. lusitaniae*, *C. parapsilosis*, and *C. tropicalis* did not differ significantly among themselves; *T. glabrata* and *C. krusei* were more resistant than all other species.

DISCUSSION

In this report, we have demonstrated that the MICs of D0870 were markedly influenced by a variety of test conditions. Endpoint definition and starting inoculum size had the most profound effects, ranging over 75,000-fold for some strains. Other test conditions had either less profound effects or no effect at all. However, even for these other variables, striking variations occurred with occasional strains. Thus, although a uniform effect was not demonstrated for pH or temperature, maintaining standards for these conditions as well may reduce the occurrence of anomalous results.

Detection of putatively resistant strains is the principal purpose for in vitro testing of infecting microorganisms. Prior to the availability of fluconazole, very few strains of *C. albicans* were identified as azole resistant (12, 13). *T. glabrata* had appeared relatively resistant in vitro under some conditions, but clinical experience with azole treatment was limited (8, 14–16, 21). More recently, there has been an increasing number of *C. albicans* strains isolated from patients failing fluconazole therapy for either mucosal or deep-seated infection (3, 23, 27). However, it is not always clear whether failure is due to intrinsic drug resistance or to host factors such as immunodeficiency, disrupted anatomy, or the presence of indwelling vascular cannulae or other prosthetic material.

Several reports have indicated that *C. krusei* has emerged during fluconazole prophylaxis as well as failing fluconazole therapy (1, 4, 17, 20, 24, 28, 30), and on the basis of these reports most authorities believe that this species is more difficult to treat than are the majority of infections due to *C. albicans*. In the present report, we have used this putative difference to evaluate various combinations of test conditions. By this strategy, our findings have indicated that the procedure specified by the NCCLS reference method produces the greatest discrimination between these two species. Although most azole resistance to date has been common to all drugs of that class, results from experimental infections in animals and clinical trials will be needed to corroborate this predicted relationship.

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