

## Disk Diffusion Method for Determining Susceptibilities of *Candida* spp. to MK-0991

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**We have developed an agar-based methodology for testing susceptibilities of *Candida* spp. to the new antifungal agent MK-0991, a glucan synthase inhibitor. Results obtained with this method correlated well with the results obtained by the National Committee for Clinical Laboratory Standards M27-A broth microdilution reference method. However, as noted with prior comparisons of broth- and agar-based systems, some isolates yielded inhibition zones which were not consistent with the MICs obtained for them. Understanding the implications of these differences will require testing in an in vivo system.**

New approaches for testing the susceptibilities of fungi continue to be developed. Agar-based methods are attractive because of their simplicity and low cost (2). In addition, they may provide an enhanced detection of resistance, as is the case with the amphotericin B susceptibilities of *Candida* spp. (9) and *Cryptococcus neoformans* (6). In this study, we have explored the use of an agar-based methodology for testing the susceptibilities of *Candida* spp. to the new water-soluble pneumocandin, MK-0991. This agent has fungicidal activity in vitro (3) and potent activity in animal models against disseminated candidiasis and aspergillosis (1) and has been shown to enhance the activities of amphotericin B and fluconazole against *C. neoformans* in vitro (4).

(This work was presented in part at the 97th General Meeting of the American Society for Microbiology in 1997 [7a].)

A collection of 94 isolates belonging to three different genera was used in this study (Table 1). The collection also included two isolates of *C. neoformans* which were used as controls for resistance because of the known lack of susceptibility of these organisms to this class of drugs both in vitro (3) and in vivo (1). All the organisms were kept at  $-70^{\circ}\text{C}$  and were passaged at least twice on Sabouraud dextrose agar at  $35^{\circ}\text{C}$  prior to being tested.

A single lot of RPMI 1640 medium (lot 85H46331; Sigma Chemicals Co., St. Louis, Mo.) was used throughout the study. This culture medium was buffered with 3-(*N*-morpholino)propanesulfonic acid (MOPS) (lot 75H5734; Sigma Chemicals Co.) to achieve a final concentration of 0.165 M, and its pH was adjusted to 7.0. The final glucose concentration of the RPMI 1640 medium was 0.2% (2 g/liter). For the agar tests, a double-strength RPMI 1640 medium was supplemented with glucose to achieve a final concentration of 4% (40 g/liter). The pH was adjusted to 7.0, and the culture medium was then mixed with an equal volume of 30 g of agar per liter in sterile distilled water (Bacto Agar; Difco Laboratories, Detroit, Mich.). The final medium in agar thus contained 15 g of agar per liter and 20 g of glucose per liter.

Susceptibility determination was carried out two different ways. For the broth tests, the microdilution adaptation of M27-A (7) was performed by using twofold dilutions of MK-

0991 at concentrations ranging from 0.0078 to 4  $\mu\text{g/ml}$ . MICs were determined by measuring the optical density at 530 nm with a plate reader (model EL-310; BIO-TEK, Burlington, Vt.) after both 24 and 48 h of incubation at  $35^{\circ}\text{C}$ . The plates were agitated prior to being read, and the MIC was the lowest concentration of MK-0991 which completely inhibited fungal growth. For the agar-based methods, a diffusion disk method was used. A 0.5 McFarland suspension of each isolate (prepared per the M27-A protocol) was swabbed in three directions on RPMI 1640 medium–2% glucose agar plates. These inoculated plates were left to dry for at least 20 min, after which BBL blank paper disks (6.3-mm diameter; Becton Dickinson Microbiology Systems, Cockeysville, Md.) previously saturated with 25  $\mu\text{l}$  of a solution containing 100  $\mu\text{g}$  of MK-0991 per ml were placed on the plates. The final concentration was 2.5  $\mu\text{g/disk}$ . This concentration was selected based on preliminary tests and yielded the best range of zone diameters of all the concentrations tested (50, 100, and 200  $\mu\text{g/ml}$ ). Zone diameters (in millimeters) for the zone of complete inhibition were determined after 24 and 48 h of incubation at  $35^{\circ}\text{C}$  and compared with MICs determined by the National Committee for Clinical Laboratory Standards M27-A microdilution method. Under these conditions, good growth was obtained for all tested isolates. Zone edges were sharply defined and easily determined.

Table 1 shows the activity of the compound against the 94 isolates sorted by species. A tendency towards slightly smaller zones and increased MICs was apparent after 48 h of incubation, but results after 24 and 48 h of incubation were otherwise qualitatively similar, as were the results obtained upon repeat testing of a subset of the isolates (data not shown). Consistent with previous reports (1, 3), the two *C. neoformans* isolates were not inhibited by MK-0991 as manifested by MICs of  $>4$   $\mu\text{g/ml}$  and no zone of inhibition.

Figure 1 shows the isolate-by-isolate relationship between  $\log_{10}$  MIC and the inhibition zones produced on agar after 24 and 48 h of incubation, respectively. For most organisms tested, the more susceptible the organism was by M27-A, the greater the inhibition zone that was produced on agar. Thus, for the isolates that had the smallest zone diameters M27-A broth MICs were always elevated compared with the median MIC. However, the converse was not always true: we noted a few isolates for which MICs were elevated that did not have small inhibition zones after either 24 or 48 h of incubation. These include one isolate of *Candida glabrata* for which the

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TABLE 1. Susceptibility data obtained with the two methodologies<sup>a</sup>

Incubation time (h)	Species	No. of isolates	Inhibition zone (mm)		MIC ( $\mu\text{g/ml}$ )	
			Mean	Range	Geo mean	Range
24	<i>C. albicans</i>	45	15.9	14–19	0.44	0.25–4
	<i>C. tropicalis</i>	10	15.2	13.5–17	0.8	0.5–2
	<i>C. glabrata</i>	10	16.9	15–18	1.2	0.5–2
	<i>C. parapsilosis</i>	10	13.2	9.5–16	2	0.5–>4
	<i>C. krusei</i>	9	14.6	12–17	2	2
	<i>C. lusitaniae</i>	6	12.7	11–14	2.6	1–4
	<i>C. lipolytica</i>	1	12.5		2	
	<i>S. cerevisiae</i>	1	13		2	
	<i>C. neoformans</i>	2	No zone	No zone	>4	>4
48	<i>C. albicans</i>	45	15.6	14–19	0.47	0.25–4
	<i>C. tropicalis</i>	10	14.9	13.5–17	1.1	0.5–4
	<i>C. glabrata</i>	10	16.9	15–18	1.7	0.5–2
	<i>C. parapsilosis</i>	10	12	9.5–16	2	2–>4
	<i>C. krusei</i>	9	14.1	12–17	2	2–>4
	<i>C. lusitaniae</i>	6	11.8	11–14	2.6	2–4
	<i>C. lipolytica</i>	1	12.5		2	
	<i>S. cerevisiae</i>	1	13		2	
	<i>C. neoformans</i>	2	No zone	No zone	>4	>4

<sup>a</sup> Geo mean, geometric mean MIC. No zone, no inhibition zone was seen.

MIC was 2  $\mu\text{g/ml}$  (24 and 48 h) but with a zone of 18 mm (24 and 48 h), one isolate of *Candida krusei* for which the MIC was 2  $\mu\text{g/ml}$  (24 and 48 h) but with zones of 17 mm (24 h) and 16 mm (48 h), and one isolate of *C. krusei* for which the MICs were 2  $\mu\text{g/ml}$  (24 h) and >4  $\mu\text{g/ml}$  (48 h) but with zones of 17 mm (24 h) and 16 mm (48 h). In addition, for two other isolates of *C. glabrata* the 24-h-incubation MIC was 1  $\mu\text{g/ml}$  and the 48-h-incubation MIC was 2  $\mu\text{g/ml}$ , but zone diameters remained stable at 17 to 18 mm at both time points.

To our knowledge, this is the first report on MK-0991 to demonstrate a correlation between the in vitro susceptibility data from a broth microdilution technique and an agar-based methodology. For most isolates, zone diameters for MK-0991 on RPMI 1640 medium–2% glucose agar rose predictably as M27-A-determined MICs fell. In agreement with the work of others (5, 8), we observed that MICs varied according to species and that two distinct groups of species could be identified.

The first group contained *Candida albicans*, *Candida tropicalis*, and *C. glabrata* and was more susceptible than the group containing *Candida lusitaniae*, *C. krusei*, and *Candida parapsilosis*. When analyzed with the agar zone diameter data, this same susceptibility gradient was again evident. This general pattern was seen with both the 24- and the 48-h-incubation data. MICs were consistently the highest for the *C. lusitaniae* isolates (geometric mean, 2.6  $\mu\text{g/ml}$  at both 24 and 48 h), and these isolates had the smallest inhibition zones on agar (12.7 and 11.8 mm after 24 and 48 h, respectively), suggesting the possibility of a strain-specific reduced susceptibility to MK-0991. For one isolate each of *Candida lipolytica* and *Saccharomyces cerevisiae* MICs were also elevated and zone diameters were small, but additional isolates of these species need to be tested before any conclusions can be drawn.

In summary, we found that an MK-0991 MIC range of 0.25 to 1  $\mu\text{g/ml}$  comprised 71 and 63% of all the isolates tested after

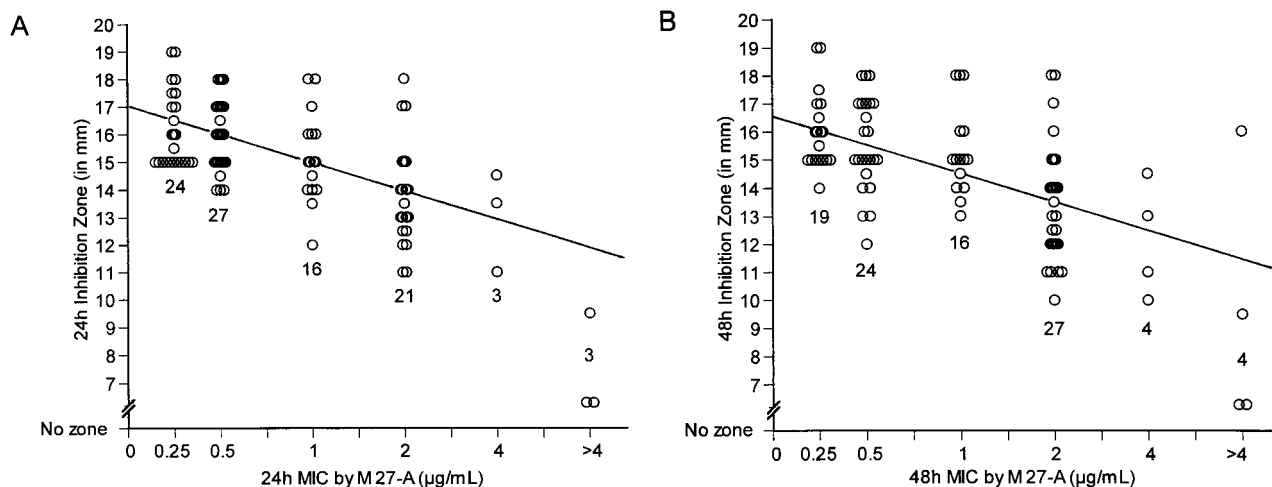


FIG. 1. Correlation of broth method-determined MICs with agar inhibition zones. MK-0991 susceptibility values for 94 isolates obtained by the broth microdilution M27-A methodology versus the corresponding inhibition zones on agar are shown at 24 h (A) and 48 h (B). The line of best fit is shown, and the regression statistics are as follows:  $y = 14.77 - 2.96 \log_{10} [\text{MIC}]$  ( $r = 0.59$ ) at 24 h and  $y = 14.59 - 2.83 \log_{10} [\text{MIC}]$  ( $r = 0.53$ ) at 48 h. Shown also is the number of isolates at each MIC.

24 and 48 h, respectively. Bartizal et al. (3) recently reported a similar range for a collection of isolates which included six of the seven *Candida* species we present here. The range of the inhibition zones produced for this MIC range was 12 to 19 mm for both 24 and 48 h. Based on the distribution of MICs for the entire study population, isolates for which MICs and zone diameters were in this range should be judged as susceptible to MK-0991. The clinical implications of smaller zone diameters and larger MICs will require further investigation, as will the disparity between these two measures for some isolates.

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