Determination of Echinocandin MICs for *Candida* Species in Less than 8 Hours: Comparison of the Rapid Susceptibility Assay with the Clinical and Laboratory Standards Institute's Broth Microdilution Assay[▽]

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Received 3 July 2009/Returned for modification 26 August 2009/Accepted 9 October 2009

The echinocandins prevent fungal cell wall synthesis by inhibiting β -1,3-glucan synthesis, a significant glucose-consuming process. Previous studies suggested that echinocandin inhibitory activity is evident within 1 h of exposure. We hypothesized that a susceptibility assay based on glucose consumption may provide clinically useful MICs rapidly. The rapid susceptibility assay (RSA), which provides MICs in less than 8 h, was compared with the standard broth microdilution susceptibility assay (Clinical and Laboratory Standards Institute, document M27-A3, 2008) for 56 *Candida* species strains. Variables which are known to influence MICs determined by the M27-A3 method were also assessed for their effects on the RSA results. Excellent agreement (>90%) between the results of the RSA and M27-A3 methods was achieved for all three FDA-approved echinocandins (micafungin, caspofungin, and anidulafungin). *Candida lusitanae* strains were responsible for most of the discordant results. Assay variables such as the test medium, the age of the inoculum culture, and the presence of human serum affected MIC results from the RSA and the M27-A3 method similarly. The RSA is equivalent to the standard M27-A3 method for determining echinocandin MICs for *Candida* species. The RSA provides MIC results in less than 8 h and can be applied to old and young yeast colonies. The assay could potentially provide clinically useful MICs on the same day that yeast growth from a specimen is first detected on solid medium.

Echinocandins are the newest class of FDA-approved drugs for the treatment of systemic fungal infections and the first approved class of antifungals that target the cell wall, specifically the synthesis of β -1,3-glucan. The spectrum of fungi which are susceptible to echinocandins is narrower than the spectrum of fungi susceptible to the polyenes, despite the presence of β -1,3-glucan in the cell walls of many nonzygomycetous fungi. However, the echinocandins have proven to be excellent agents for the treatment of candidiasis. In 2004, the Infectious Diseases Society of America published a practice guideline in which echinocandins are listed as one of the first-line options for the treatment of candidemia (8).

Similar to the actions of β -lactam antibacterial agents, the inhibition of cell wall polysaccharide synthesis by echinocandins potentially results in cell death. Echinocandins inhibit β -1,3-glucan synthase (GS) in the cell membrane by targeting the Fks1p and Fks2p components of GS complexes. As a consequence, β -1,3-glucan, which is the predominant polysaccharide in the cell walls of *Candida* species, is not produced while cell wall remodeling and cell expansion continue to occur, resulting in weakness in the wall and susceptibility to cell lysis due to overwhelming turgor pressure (6). A potential outcome

of the events prior to cell death is diminution of glucose uptake or consumption.

Clancy et al. (2) have shown that cells exposed to echinocandins for only 1 h at levels in excess of the known MIC for the strain exhibit growth inhibition, as demonstrated by decreased numbers of CFU compared to those of untreated controls. This observation suggested that it may be possible to determine MICs of echinocandins for different yeast strains on the same day of testing by using the rapid susceptibility assay (RSA), especially because the RSA is based on levels of glucose consumption by cells exposed to the drug relative to those by control cells (4, 11). The RSA is similar to the Clinical and Laboratory Standards Institute (CLSI) M27-A3 broth microdilution assay except that the RSA inoculum concentration is higher, the incubation period is shorter, and the end point is based on glucose uptake measured spectrophotometrically (3, 4, 11).

The RSA was originally used by Cutler et al. (4, 11) to provide MICs of fluconazole and amphotericin in less than 24 h. Their results showed that MICs of the rapidly acting antifungal agent amphotericin B can be obtained in 6 h but that the slower-acting triazole fluconazole requires at least 8 to 18 h. The RSA has since been applied to *Aspergillus fumigatus* to obtain MICs of voriconazole, itraconazole, and amphotericin B (12, 13).

Here, we evaluate the ability of the RSA to provide echinocandin MICs equivalent to those obtained by the "gold standard" CLSI M27-A3 method, which requires an incubation period of 24 h, for *Candida* species on the same day of testing. Further, the RSA and M27-A3 method were compared to

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[▽] Published ahead of print on 21 October 2009.

determine if the MICs provided are similarly affected by variations in inoculum growth conditions, the test medium, and the presence of human serum. The results demonstrate that the RSA method is comparable to the M27-A3 method but provides MICs in less than 8 h regardless of whether inoculum cells were prepared from 4- or 1-day-old cultures.

MATERIALS AND METHODS

Yeast strains and growth conditions. Eight strains of each of seven *Candida* species (*C. albicans*, *C. glabrata*, *C. guilliermondii*, *C. krusei*, *C. lusitanae*, *C. parapsilosis*, and *C. tropicalis*) were used to compare MICs of micafungin obtained with the RSA to those obtained with the M27-A3 method. Quality control strains *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 were also included. The isolates comprised archived strains from the University of Virginia clinical microbiology laboratory, strains from the authors' laboratory archive, gifts from Merck, and strains from D. J. Diekema of the University of Iowa. This collection of isolates included 19 strains for which caspofungin MICs were known to be elevated (for these strains, the MIC-2 determined by the M27-A2 method [MIC-2_{M27}], defined as the minimal drug concentration that results in prominent [approximately 50%] reduction in growth compared to that of controls, was ≥ 1 $\mu\text{g/ml}$). When anidulafungin and caspofungin MICs determined by the RSA and the M27-A3 method were compared, a subset of 10 isolates among the 58 strains was used rather than the entire strain collection because there is no evidence that the performance of the M27-A3 assay with anidulafungin and caspofungin differs from its performance with micafungin. At least one strain of each species was included.

Unless otherwise noted, strains were subcultured twice on plates of Sabouraud dextrose agar with the Emmons modification (SDA), and each subculture was grown for 23 to 25 h in a humidified incubator with atmospheric air at 37°C. This temperature was chosen because it is the temperature used previously for the RSA and because preliminary studies with the quality control strains showed that it did not affect the M27-A3 MICs. The use of a single temperature for the subcultures helped limit possible subsequent variability in the preparation of inoculum suspensions. Stock suspensions of the strains were maintained in sterile deionized water at room temperature.

Echinocandin solutions and preparation of drug plates. Caspofungin, anidulafungin, and micafungin powders were obtained from the manufacturers (Merck and Co., Pfizer, and Astellas Pharma, respectively). Powders were solubilized in sterile deionized H₂O (for caspofungin and micafungin) or dimethyl sulfoxide (DMSO; for anidulafungin) to achieve a concentration of active drug of 5,120 $\mu\text{g/ml}$. Twenty-five-microliter aliquots of each drug stock (i.e., 128 μg of each drug) were prepared and frozen at -80°C .

To prepare microdilution plates with micafungin or caspofungin, one or more 25- μl aliquots were thawed and the volume required to obtain either 32 or 4 $\mu\text{g/ml}$ was transferred into medium herein designated RPMI-0G, consisting of RPMI 1640 medium without glutamine and without glucose (Sigma, St. Louis, MO) buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS) buffer (Sigma). Any unused thawed drug was discarded. Twofold serial dilutions in RPMI-0G were prepared. Aliquots (0.1 ml) of each of the 10 drug dilutions were transferred into the wells of plastic microdilution plates (item no. 3799; Corning Inc., Corning, NY). Microdilution plate wells in columns 11 (positive growth control) and 12 (negative control) received 0.1 ml of RPMI-0G. The trays were sealed and frozen at -20°C until needed, but no longer than 2 weeks. To prepare anidulafungin drug plates, 55 μl of DMSO was added to a thawed 25- μl aliquot and mixed and then 320 μl of RPMI-0G was added. After being mixed, the entire volume was transferred into 3.6 ml of RPMI-0G. This procedure resulted in a solution of 32 μg of drug/ml of RPMI-0G with 2% DMSO. The solution was further diluted 1:8 with RPMI-0G containing 2% DMSO to obtain a drug concentration of 4 $\mu\text{g/ml}$. Subsequent twofold dilutions with RPMI-0G–2% DMSO were made.

Susceptibility test methods. Except where noted below, RPMI 1640 medium with MOPS was used as the test medium base in all assays. RPMI 1640 medium with 0.2% glucose (RPMI-0.2G) was used for the RSA method, while RPMI 1640 medium with 0.4% glucose (RPMI-0.4G) was used for the M27-A3 method. This allowed the final glucose concentrations in the respective assays to be 0.1 and 0.2%.

To prepare the inoculum, isolated colonies from the second-subculture plates were suspended in RPMI-0.2G to achieve a turbidity approximating a 0.5 McFarland standard. This suspension was used to prepare the inoculum dilutions for both the RSA and the M27-A3 assay. Exceptions to this method of preparation are noted for specific experiments.

(i) RSA method. The RSA was performed as described elsewhere with several modifications (11). The 0.5 McFarland standard inoculum suspension was diluted 1:4 in test medium (RPMI-0.2G), and 100 μl of the dilute suspension was added to each of the drug wells and the positive control wells in quadruplicate. The negative control well received 100 μl of test medium. Once inoculated, plates were placed in a humidified incubator under atmospheric air at 37°C and incubated for 6 to 7 h. To determine the amount of glucose left in each well, an enzyme substrate color mix was added to each well with an eight-channel repeating pipette and color development was allowed to proceed for 20 min. The color mix was always added beginning with the negative control column (column 12) and progressing sequentially to the column with the highest drug concentration (column 1).

After incubation, the relative amount of glucose in each well was determined with a colorimetric glucose oxidase assay (11) and the percent residual glucose for each drug concentration relative to the level in the growth control was calculated by using the following formula: $[(A_{\text{drug}} - A_{\text{growth control}})/(A_{\text{neg control}} - A_{\text{growth control}})] \times 100$, where A is the average absorbance at a wavelength of 540 nm, A_{drug} is the value for the wells containing the drug, $A_{\text{growth control}}$ and $A_{\text{neg control}}$ are the values for the growth control and the negative control, and $(A_{\text{neg control}} - A_{\text{growth control}})$ is the glucose consumption dynamic range.

For each drug-yeast strain combination, RSA results were determined based on the percent residual glucose and the glucose consumption profiles. However, the definitions of the measures obtained by the RSA are modified from the criteria presented in CLSI document M27-A3 (3). The MIC-0 determined by the M27-A3 method (MIC-0_{M27}) is defined as the minimal concentration of the drug that results in complete inhibition of growth. The MIC-0 determined by the RSA (MIC-0_{RSA}) is the lowest drug concentration at which no further inhibition of glucose consumption with higher drug concentrations was seen (that is, the lowest concentration at which the inhibition curve becomes a plateau at the highest percent residual glucose). The MIC-2_{M27} is defined as the minimal drug concentration that results in prominent (approximately 50%) reduction in growth compared to that of controls. The MIC-2 determined by the RSA (MIC-2_{RSA}) is defined as the lowest concentration at which 50 to 85% of the residual glucose obtained at the MIC-0 value was obtained (for example, if the residual percent glucose at the MIC-0_{RSA} was 80%, then the MIC-2_{RSA} is the lowest concentration at which 40 to 70% residual glucose was observed).

(ii) Broth microdilution assay. Broth microdilution susceptibility assays were performed as described in CLSI document M27-A3, with some modifications (3). The first modification is that the drug dilution scheme was serial twofold dilution. The second is that the inoculum was prepared in RPMI-0.2G, not sterile water. This was done to accommodate the inoculum dilution used for the RSA and to ensure that both susceptibility assays used the same biological population. The two-step dilution scheme (total dilution, 1:1,000) for the 0.5 McFarland standard suspension to prepare the inoculum described in CLSI document M27-A3 was followed except that both diluents were RPMI-0.4G.

Inoculated multiwell plates were incubated for 24 h at 35°C. Turbidity was typically determined by subjective observation, but in some cases measurement was achieved spectrophotometrically to confirm subjective measurements. In the inoculum age experiments described below, final turbidity was measured spectrophotometrically. The MIC-0_{M27} and MIC-2_{M27} values were determined in accordance with CLSI M27-A3 guidelines (3).

MIC comparisons and evaluation of discrepant results. The MIC-0_{RSA} and MIC-2_{RSA} values were each compared with the MIC-0_{M27} and MIC-2_{M27} values. This was done in order to determine how well and to what extent the RSA method behaves like the M27-A3 method. Values were considered to be in agreement if the RSA MIC was within one doubling dilution of the M27-A3 MIC. Any strain that produced one or more sets of discrepant results was retested, and if the originally discrepant results became concordant upon retesting, an additional repeat test was performed. If results from both repeat tests were in agreement, then the final results were considered to be in agreement.

Am3 as test medium. Antibiotic medium 3 (Am3) broth (Becton, Dickinson and Co., Sparks, MD) supplemented with 0.1% glucose for a final glucose concentration of 0.2% (Am3-0.2G) was prepared as described by the manufacturer and filter sterilized. To compare the effect of the test medium (RPMI 1640 medium versus Am3) on MICs obtained by the RSA and the M27-A3 method without the confounding effect of inoculum concentration differences, the starting 0.5 McFarland standard suspension was prepared in sterile deionized water. This suspension was then diluted into the standard test media (Am3-0.2G and RPMI-0.4G) for the M27-A3 assays. For the RSA, the suspension was diluted 1:4 in 1.33 $\times$ Am3-0.2G and 1.33 $\times$ RPMI-0.4G. In these experiments, a subset of the initial 58 yeast strains was tested. This subset was composed of two strains from each of the seven species.

FIG. 1. Glucose consumption profiles produced by caspofungin-susceptible strains (*C. albicans* Mk-A and *C. krusei* Mk-A) and a resistant strain (*C. albicans* NR3). The MIC-0 and MIC-2 for *C. albicans* Mk-A are 1 µg/ml, while those for *C. krusei* Mk-A are 2 µg/ml. The glucose consumption profile for *C. albicans* NR3 does not plateau at the highest drug concentrations, suggesting that the MIC-0 for this strain is ≥ 8 µg/ml. Test medium, RPMI-0.1G. conc., concentration.

Human serum supplementation. Frozen human serum aliquots (Bioreclamation Inc., Westbury, NY) were thawed and clarified by centrifugation at 5,000 × g for 10 min. The serum was added to Am3-0.2G (test medium for the RSA) to achieve a concentration of 26.6%, and the mixture served as the diluent for the 0.5 McFarland standard cell suspension which was prepared in Am3-0.2G. The glucose concentration in the serum was assayed by the University of Virginia Medical Laboratories.

RESULTS

Initial RSA development. A caspofungin-susceptible isolate (strain Mk-A) and a resistant isolate (strain NR3) of *C. albicans* (provided by C. Douglas of Merck Research Laboratories) were used for initial RSA development. The resistant

isolate has a mutation in the gene *FKSI*, which encodes a major protein component of GS, rendering GS less sensitive to caspofungin (5, 9). Caspofungin and RPMI 1640 medium with different glucose concentrations (0.05, 0.1, and 0.2%, wt/vol) were used for developing the initial RSA conditions required for echinocandin susceptibility testing of *Candida* species. An incubation period of 8 h was selected based on our previous experience with testing fluconazole and amphotericin B by the RSA and based on the report of Clancy et al. (2) demonstrating that a 1-h exposure period is sufficient for caspofungin to inhibit yeast growth. A broad glucose consumption dynamic range (that is, the difference between the amount of glucose consumed by untreated cells and the initial amount of glucose) was obtained by using a final inoculum equivalent to an eight-fold dilution of a 0.5 McFarland turbidity standard and RPMI-0.1G. An example of the types of curves that may be seen with the RSA is shown in Fig. 1. Subsequent experiments demonstrated that broad dynamic ranges could be achieved with a 6-h echinocandin exposure period. For the purposes of the present study, the incubation period varied between 6.5 and 7.25 h.

Comparison of micafungin MICs obtained by the RSA and the M27-A3 method. The susceptibilities of eight strains of seven *Candida* species to micafungin were tested by the RSA method and the M27-A3 method, and the MIC-0 and MIC-2 results for these strains were compared. Complete agreement was obtained with four species (Table 1). The poorest agreement was seen when the MIC-0_{RSA} was compared with the MIC-2_{M27}. When the MIC-2_{RSA} was compared with the MIC-2_{M27}, only 4 of 56 isolates showed discordance. All four isolates were *C. lusitaniae* strains. Of these four, two had results discordant by only two twofold drug dilutions. Of the total of 21 discordant sets of results, 16 were associated with the *C. lusitaniae* isolates. Only two instances occurred in which the original discordant results were changed to concordant results after repeat testing. Both involved the comparison of MIC-2_{RSA} with MIC-0_{M27}.

TABLE 1. Concordance of micafungin MIC-0 and MIC-2 values obtained by the RSA and M27-A3 methods for 56 isolates of *Candida* species

Species (no. of isolates tested) or isolate group	Range (µg/ml) for:				No. with results in agreement/no. tested (% agreement) or no. with discrepant results for:			
	MIC-0 _{RSA}	MIC-2 _{RSA}	MIC-0 _{M27}	MIC-2 _{M27}	MIC-0 _{RSA} vs MIC-0 _{M27}	MIC-0 _{RSA} vs MIC-2 _{M27}	MIC-2 _{RSA} vs MIC-0 _{M27}	MIC-2 _{RSA} vs MIC-2 _{M27}
<i>C. albicans</i> (8)	≤ 0.03 –2	≤ 0.03 –2	≤ 0.03 –2	≤ 0.03 –1	8/8	8/8	8/8	8/8
<i>C. guilliermondii</i> (8)	0.125–1	0.125–0.5	0.25–1	0.625–0.25	8/8	5/8	8/8	8/8
<i>C. glabrata</i> (8)	≤ 0.03	≤ 0.03	≤ 0.03	≤ 0.03	8/8	8/8	8/8	8/8
<i>C. parapsilosis</i> (8)	0.5–2	0.125–0.5	0.25–1	0.125–0.5	8/8	7/8	7/8	8/8
<i>C. krusei</i> (8)	0.0625–2	0.0625–2	0.0625–2	0.03125–1	8/8	8/8	8/8	8/8
<i>C. tropicalis</i> (8)	≤ 0.031 –1	≤ 0.031 –0.5	≤ 0.031 –0.5	≤ 0.031 –0.5	8/8	8/8	8/8	8/8
<i>C. lusitaniae</i> (8)	≤ 0.031 –0.5	≤ 0.031 –0.5	≤ 0.031 –0.125	≤ 0.031 –0.0625	3/8	3/8	6/8	4/8
All species (56)	≤ 0.03 –2	≤ 0.03 –2	≤ 0.03 –2	≤ 0.03 –1	51/56 (91.1) ^a	47/56 (83.9)	53/56 (94.6)	52/56 (92.9) ^a
Median	0.125	0.125	0.0625	0.0625				
Mode	≤ 0.03	≤ 0.03	≤ 0.03	≤ 0.03				
Isolates with results differing by:								
2 doubling dilutions					2	5	3	2
3 doubling dilutions					2	3	0	2
>3 doubling dilutions					1	1	0	0

^a All discrepant results were due to *C. lusitaniae* isolates.

TABLE 2. Results from the RSA versus those from the M27-A3 method for caspofungin and anidulafungin

Drug	Range ($\mu\text{g/ml}$) for:				No. of pairs in agreement/no. of comparisons of:			
	MIC-0 _{RSA}	MIC-2 _{RSA}	MIC-0 _{M27}	MIC-2 _{M27}	MIC-0 _{RSA} vs MIC-0 _{M27}	MIC-0 _{RSA} vs MIC-2 _{M27}	MIC-2 _{RSA} vs MIC-0 _{M27}	MIC-2 _{RSA} vs MIC-2 _{M27}
Caspofungin	0.25–1	0.25–1	0.5–4	0.25–1	9/10 ^a	10/10	9/10 ^a	10/10
Anidulafungin	≤ 0.0039 – ≥ 4	≤ 0.0039 – ≥ 4	≤ 0.0039 – ≥ 2	≤ 0.0039 – ≥ 1	10/10	8/10 ^a	10/10	9/10 ^a

^a Each pair of results not in agreement was due to MICs differing by two doubling dilutions. One yeast strain (*C. guilliermondii* IAW-12) was responsible for four of the five sets of discordant results.

Throughout this phase of the study, the quality control strain *C. parapsilosis* ATCC 22019 was run with each set of three test strains (producing 21 occurrences). The range of MIC-0_{RSA} values was 0.5 to 1.0 $\mu\text{g/ml}$, while the range of MIC-2_{RSA} values was 0.25 to 0.5 $\mu\text{g/ml}$, indicating excellent run-to-run consistency.

Comparison of RSA and M27-A3 MICs of caspofungin and anidulafungin. Two strains each of *C. albicans*, *C. glabrata*, *C. krusei*, *C. lusitanae*, and *C. parapsilosis* were used to compare the performance of the RSA against that of the M27-A3 method for the echinocandins caspofungin and anidulafungin. For both echinocandins, the two susceptibility methods showed good agreement (Table 2). Of the total of five sets of discordant results, four were due to a single *C. guilliermondii* strain. The fifth pair of discordant results was due to *C. albicans* strain IAW-20. In all instances in which discordance occurred, the difference was two twofold drug dilutions.

Effect of test medium. Am3 has been reported to yield lower echinocandin MICs than RPMI 1640 medium when the drugs are tested in the standard M27-A assay (1, 10). To confirm whether the RSA method behaves equivalently, 13 yeast strains (2 strains each of *C. albicans*, *C. glabrata*, *C. guilliermondii*, *C. krusei*, and *C. tropicalis* and 3 strains of *C. lusitanae*) were tested. Most of the strains used in this comparison had produced the discordant results indicated in Table 1. These strains were used in order to determine if Am3-0.2G might improve agreement between the results of the RSA and the M27-A3 method.

Am3-0.2G caused lower MIC-0_{RSA} and MIC-2_{RSA} values than RPMI-0.1G for all 13 isolates (Table 3). When Am3-0.2G was compared with RPMI-0.2G in the M-27A assay, three instances of similar MICs were obtained. However, in two cases, the similar MICs were due to the limit of the lowest drug concentration used in the comparison. Therefore, it is possible that Am3-0.2G may have resulted in lower MICs than RPMI-0.2G if additional drug dilutions had been tested.

Levels of agreement between the RSA and M27-A3 MICs were better with Am3 than with RPMI 1640 medium ($P = 0.025$; one-tailed test of proportions). Eleven pairs of discordant MICs were obtained with RPMI 1640 medium, while only four occurred with Am3. Three of the discordant MIC pairs obtained with Am3 also occurred with RPMI 1640 medium.

Effect of inoculum preparation. Preliminary studies in which cells were grown in suspension at 37°C in Am3-0.2G to exponential and stationary phases demonstrated that the age of the cells did not affect the MIC-0 obtained with the RSA but did affect the glucose consumption dynamic range. The CLSI document M27-A3 recommends that inoculum cells be grown on SDA for 24 h and that isolated colonies be utilized to prepare the test inoculum, suggesting that variance from these practices may result in altered MICs. To test whether MICs obtained with

the M27-A3 method and the RSA are affected differently by inoculum age and crowding, cells were inoculated onto SDA plates 24 and 96 h prior to testing such that isolated colonies would be obtained as well as areas of confluent growth. Inocula were prepared using cells from these two growth patterns. M27-A3 MICs were determined spectrophotometrically.

MIC-0 and MIC-2 values from the RSA and the M27-A3 method were not affected by the age of the cells used to prepare the inocula (Table 4). The abilities of 4-day-old cells derived from single colonies to consume glucose during the 7-h RSA incubation period and to grow during the 24-h M27-A3 incubation period were generally reduced compared to those of 1-day-old cells. In the RSA, inocula prepared from confluent colonies consumed less glucose than inocula from single colonies, regardless of the growth incubation period. However, a similar difference in growth turbidity between the two inoculum types in the M27-A3 method was not observed.

Effect of human serum on MIC-0_{RSA}. Paderu et al. (7) reported that the addition of human serum (50%, vol/vol) to RPMI-0.2G used in the CLSI M27-A3 broth microdilution method resulted in the elevation of MICs of the three commercially available echinocandins. We tested whether glucose consumption is affected by the presence of human serum and therefore whether the RSA behaves equivalently to the CLSI M27-A3 method. Serum had little effect on the consumption

TABLE 3. Comparison of effects of test medium on RSA versus M27-A3 MIC results^a

Measure or comparison	Range ($\mu\text{g/ml}$) determined with:		No. of isolates with results in agreement/no. tested in:		No. of Am3 results lower than RPMI 1640 medium results/no. of comparisons
	RPMI 1640 medium	Am3	RPMI 1640 medium	Am3	
MIC-0 _{RSA}	0.0079–1	≤ 0.0039 –0.5			13/13
MIC-2 _{RSA}	0.0079–0.5	≤ 0.0039 –0.25			13/13
MIC-0 _{M27}	≤ 0.0039 –0.5	≤ 0.0039 –0.25			12/13 ^b
MIC-2 _{M27}	≤ 0.0039 –0.5	≤ 0.0039 –0.25			
MIC-0 _{RSA} vs MIC-0 _{M27}			11/13	12/13	11/13 ^c
MIC-0 _{RSA} vs MIC-2 _{M27}			6/13	11/13	
MIC-2 _{RSA} vs MIC-0 _{M27}			13/13	12/13	
MIC-2 _{RSA} vs MIC-2 _{M27}			11/13	10/13	

^a Comparisons were made to determine if Am3-0.2G causes effects on RSA MICs similar to those on M27-A3 MICs relative to MICs obtained with RPMI 1640 medium. Isolates used for these comparisons were responsible for the discordant results indicated in Table 1.

^b Due to one comparison in which both MICs were ≤ 0.0039 $\mu\text{g/ml}$.

^c Due to one comparison in which both MICs were ≤ 0.0039 $\mu\text{g/ml}$ and a second comparison in which both MICs were 0.25 $\mu\text{g/ml}$.

TABLE 4. Effects of inoculum growth conditions on MICs obtained with the RSA and the M27-A3 assay^a

Organism	Inoculum type ^b	% Consumption of available glucose	Growth turbidity dynamic range (A_{590})	MIC or range of MICs ($\mu\text{g/ml}$) obtained for the four inoculum types by:			
				RSA		M27-A3 method	
				MIC-0	MIC-2	MIC-0	MIC-2
<i>C. tropicalis</i> IAW-10	1-day-old single colonies	56.75	0.328				
	1-day-old confluent colonies	24.81	0.138	0.5	0.25	0.25	0.25
	4-day-old single colonies	39.79	0.25				
	4-day-old confluent colonies	32.70	0.289				
<i>C. albicans</i> IAW-20	1-day-old single colonies	56.98	0.192				
	1-day-old confluent colonies	43.14	0.143	0.0625–0.125	0.0625	0.0625	0.031
	4-day-old single colonies	37.70	0.121				
	4-day-old confluent colonies	13.74	0.152				
<i>C. albicans</i> A9	1-day-old single colonies	48.33	0.207				
	1-day-old confluent colonies	33.63	0.147	0.0078–0.0156	0.0078	0.0078	≤ 0.0039 –0.0078
	4-day-old single colonies	51.03	0.197				
	4-day-old confluent colonies	17.69	0.291				
<i>C. glabrata</i> Y3.679	1-day-old single colonies	52.7	0.374				
	1-day-old confluent colonies	34.84	0.394	0.0156	0.0078	0.0078	0.0078
	4-day-old single colonies	36.51	0.392				
	4-day-old confluent colonies	31.37	0.386				
<i>C. guilliermondii</i> 8.58	1-day-old single colonies	45.5	0.248				
	1-day-old confluent colonies	32.6	0.206	0.25	0.0625–0.125	0.25	0.0625–0.125
	4-day-old single colonies	19.7	0.165				
	4-day-old confluent colonies	13	0.15				

^a All MICs are those of micafungin.^b Inoculum cells were grown for 24 h and for 96 h. On the day of testing, cells from single colonies and from confluent colonies were harvested separately and compared for the effects of the growth condition on MIC outcomes.

patterns of the species tested in the present study, with the exception of *C. glabrata* isolates, except to shift the curves toward higher drug concentrations. The shifts were most pronounced with anidulafungin and micafungin in both RPMI-0.1G and Am3-0.2G. The presence of human serum caused the consumption curves for *C. glabrata* isolates to become distorted, especially at low drug concentrations. The distortion was due in part to the relatively poor glucose consumption by the growth control cells, thus magnifying point-to-point variation.

The presence of human serum in the test medium increased MIC-0_{RSA} values for micafungin a minimum of 8-fold, those

for anidulafungin a minimum of 32-fold, and those for caspofungin a minimum of 1-fold, with a maximum increase of 8-fold (Table 5). The increases in the MIC-0_{RSA} values for micafungin and anidulafungin were typically 32- to 64-fold or higher. These results suggest that caspofungin is less susceptible than micafungin and anidulafungin to the effects of serum on its inhibitory activity when tested in the RSA (Table 2). This finding is consistent with the results obtained by Paderu et al. by the M27-A3 method. Unlike the studies of Paderu et al. (7), our study allowed us to assess the effects of human serum on the responses of one *C. glabrata* isolate to echinocandins by using the

TABLE 5. Evaluation of the effect of 10% human serum in Am3-0.2G on echinocandin MIC-0_{RSA} values for *Candida* species^a

Species (no. of isolates tested)	Micafungin			Anidulafungin			Caspofungin		
	MIC-0 _{RSA} ($\mu\text{g/ml}$)		Range of increase (n-fold)	MIC-0 _{RSA} ($\mu\text{g/ml}$)		Range of increase (n-fold)	MIC-0 _{RSA} ($\mu\text{g/ml}$)		Range of increase (n-fold)
	Without serum	With 10% serum		Without serum	With 10% serum		Without serum ^b	With 10% serum	
<i>C. glabrata</i> (6)	0.0312–0.0625	2–>4	≥ 64	0.00782–0.0156	>8	>1,024	0.0156 (1)	0.0156	1
<i>C. dubliniensis</i> (6)	0.0312–1	1–>4	8–64	0.00195–0.00391	0.0625–0.25	32–64	0.0078–0.0156 (4)	0.0312–0.0625	2–4
<i>C. albicans</i> (10)	0.0312–0.25	1–8	16–32	0.000977–0.0312	0.125–4	64–128	0.0078–0.0156 (4)	0.0312–0.0625	4
<i>C. krusei</i> (4)	0.25	16	64	0.00781–0.0156	1–2	64–128	0.0625–0.5	0.25–0.5	1–4
<i>C. parapsilosis</i> (5)	1–2	>16	≥ 16	0.125–1	16–>16	≥ 32	0.125–0.5 (4)	0.5–4	4–8
<i>C. tropicalis</i> (5)	0.0312–1	1–>16	32–64	0.00195–0.0625	0.125–8	32–128	ND	ND	
<i>C. guilliermondii</i> (1)	0.5	16	32	0.125	8	64	ND	ND	
<i>C. lusitanae</i> (1)	0.125	16	128	0.0156	2	128	ND	ND	

^a The evaluation was carried out to determine if the presence of human serum causes MICs determined by the RSA to increase, as has been shown previously to occur with the M27-A3 method (7). Am3-0.2G rather than RPMI 1640 medium was used as the test medium because Am3-0.2G allows for the broadest MIC range to test the effect of serum on apparent MICs.^b The number in parentheses is the number of isolates tested if it differs from the number tested with micafungin and anidulafungin.

RSA format. That isolate, when exposed to serum, did not show uninterpretable distortion of its glucose consumption curve and produced glucose consumption dynamic ranges of at least 0.3 absorbance (A_{540}) units in both experiments.

Serum used in these studies contained 104 mg glucose/dl (normal range, 74 to 99 mg/dl). Thus, the concentration of glucose in RPMI-0.1G and RPMI-0.1G supplemented with 10% serum was the same, while in Am3-0.2G supplemented with 10% serum, the glucose concentration was 0.19% instead of the 0.2% present in unsupplemented Am3-0.2G.

DISCUSSION

The RSA measures the amount of glucose used by cells exposed to echinocandins relative to that used by cells not exposed to the drug. The measurement is a result primarily of the inhibition of glucose utilization by GS by echinocandins and secondarily of the inhibition of cell growth and metabolism caused by aberrant cell wall formation. While it is possible to detect the inhibition of glucose consumption by cells exposed to echinocandins for only 2 h (data not shown), incubation for 6 to 8 h was necessary in order to obtain a broad dynamic range needed to distinguish differences in the levels of glucose consumption by cells of all seven species exposed to a range of echinocandin concentrations.

For the M27-A3 method, the MIC-2 represents an organism's relative susceptibility to an echinocandin. The best agreement ($\geq 90\%$) between the RSA and M27-A3 MIC-0 and MIC-2 values was obtained when the MIC-2_{RSA} was used (Table 1). This finding was likely due to how the MIC-0_{RSA} was defined (the lowest drug concentration at which maximum inhibition of glucose consumption became unchanged). If the measure was redefined as the concentration at which the glucose consumption inhibition curve begins to flatten prior to reaching the inhibition plateau, the nine sets of discordant results for MIC-0_{RSA} versus MIC-2_{M27} would have been reduced to five. All five would be due to *C. lusitaniae* strains. Interestingly, if the RSA MIC for those isolates was defined as the lowest concentration at which $>5\%$ inhibition of glucose consumption occurred, four of the five MICs would have been concordant with the MIC-2_{M27}. These results suggest that *C. lusitaniae* strains respond more slowly than other *Candida* species to echinocandins, resulting in the more gradual inhibition curves than those obtained with other species.

Of the seven discordant pairs of values obtained when the MIC-2_{RSA} was used for comparison to the M27-A3 method, six were due to *C. lusitaniae* strains. Therefore, if *C. lusitaniae* strains had not been used in the analysis, agreement between the MIC-2_{RSA} and MIC-0_{M27} and between the MIC-2_{RSA} and MIC-2_{M27} would have been 97.9 and 100%, respectively.

The RSA also showed patterns characteristic of the M27-A3 method. The RSA worked well for all three FDA-approved echinocandins and also yielded lower MICs with Am3-0.2G as the test medium than with RPMI-0.1G. The presence of human serum caused RSA MICs to increase, as has been reported previously for the M27-A method (7). When the inoculum age and culture density were tested, MICs obtained with the RSA and the M27-A3 method were similar. That result suggests that the M27-A3 specifications for two 24-h subcultures prior to testing and the use of isolated colonies for preparation of the 0.5 McFarland standard inoculum may not be necessary to obtain clin-

ically useful MICs. Moreover, the RSA could provide a useful MIC within 8 h after the detection of growth on a primary culture plate, regardless of whether the detected growth was in the form of isolated colonies or confluent colonies.

In summary, performance of the RSA, which allows determination of echinocandin MICs within 8 h, is equivalent to that of the M27-A3 method. Assuming that drug plates are pre-made and frozen, the clinical microbiology laboratory could report clinically useful echinocandin MICs on the first day on which growth of the isolate is detected on the primary medium used to culture a specimen. Riesselman et al. (11) have shown previously that the RSA can rapidly provide MICs of fluconazole and amphotericin B for *Candida* species. Thus, the RSA represents a substitute method for the M27-A3 assay that would help physicians modify empirical therapy to the appropriate antifungal agent for infections caused by *Candida* species sooner than is the case in current practice.

ACKNOWLEDGMENTS

This study was supported in part by funding from Merck and Co.

We thank Merck and D. J. Diekema and M. A. Pfaller of the University of Iowa for providing isolates. The assistance of the medical technologists in the University of Virginia clinical mycology laboratory is also appreciated.

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