Modification of Rapid Susceptibility Assay for Antifungal Susceptibility Testing of *Aspergillus fumigatus*

Tracy J. Wetter,^{1,3*} Kevin C. Hazen,² and Jim E. Cutler³

Department of Microbiology, Montana State University, Bozeman, Montana 59718¹; Department of Pathology, University of Virginia Health System, Charlottesville, Virginia 22908²; and Research Institute for Children, Children's Hospital, New Orleans, Louisiana 70118³

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To improve objectivity and speed of current antifungal mold susceptibility testing, the yeast Rapid Susceptibility Assay (RSA) was adapted for *Aspergillus* species. The RSA is based on glucose utilization in the presence of an antifungal drug. *Aspergillus fumigatus* conidia were incubated in 0.2% glucose RPMI 1640 containing 0.03 to 16 μ g of amphotericin B or itraconazole/ml. Drug-related inhibition of glucose utilization correlated with suppression of conidial germination. Following incubation of conidia with various concentrations of antifungal drug, the percentage of residual glucose in the growth medium was determined colorimetrically and plotted against drug concentration to determine the MIC (MIC_{RSA}). National Committee for Clinical Laboratory Standards (NCCLS) M38-P testing was also performed to obtain NCCLS MICs (MIC_{NCCLS}) for direct comparison with MIC_{RSA} s. Conidial inocula of an optical density at 530 nm (OD_{530}) of 0.11 facilitated determination of amphotericin B and itraconazole MIC_{RSA} s at 16 h equal to or within a single twofold dilution of MIC_{NCCLS} s obtained at 48 h. Preliminary testing with a 0.11- OD_{530} conidial inoculum of the slower-growing *Aspergillus terreus* resulted in itraconazole and amphotericin B MIC_{RSA} s at 16 h equal to or within a single twofold dilution of MIC_{NCCLS} s obtained at 48 h. These data indicate that the mold RSA provides a more objective and rapid method for *Aspergillus* spp. susceptibility testing than the NCCLS M38-P assay.

The rapid susceptibility assay (RSA) was previously developed by our laboratory for antifungal susceptibility testing of yeasts such as *Candida albicans* (14). The assay is based on the assumption that when fungi are exposed to an inhibitory concentration of an antifungal drug, their uptake of nutrients, such as glucose, is suppressed. A MIC (MIC_{RSA}) is determined by comparing the residual glucose levels in the medium following incubation of the fungus with and without the drug. This approach provides objective, quantitative MICs. The MIC_{RSA} s for yeast compare favorably with the National Committee for Clinical Laboratory Standards (NCCLS) M27-A antifungal susceptibility assay, but the latter uses a subjective endpoint of growth inhibition and requires more time (12).

Several compelling reasons favor adapting the RSA for use in mold susceptibility testing. First, the incidence of morbidity and mortality due to infections caused by opportunistic filamentous fungi, such as *Aspergillus fumigatus*, is increasing (4). Second, emergence of resistance during antifungal therapy has been documented (2, 11). Third, the introduction of voriconazole (5), caspofungin acetate (15), and liposomal formulations of amphotericin B has increased therapy options. Fourth, current susceptibility testing for filamentous fungi, such as the NCCLS M38-P assay, suffers from the same limitations as those cited for the yeast M27-A assay (13).

To address these issues, we have modified the RSA to facilitate susceptibility testing for *A. fumigatus*. Conidia were chosen as the inoculum type, in accordance with other susceptibility tests, with additional modifications of the initial glucose

concentration, inoculum concentration, and MIC determination. Glucose utilization was monitored during conidial germination and hyphal development in the presence or absence of amphotericin B (AMB) and itraconazole (ITC), with AMB and ITC MIC_{RSA} determinations based on the percent residual glucose. To determine whether the RSA has advantages over the NCCLS M38-P assay in terms of time and objectivity, AMB and ITC MIC_{RSA} s obtained at 16, 24, and 48 h were compared to MIC_{NCCLS} s determined at 48 h according to the NCCLS M38-P guidelines. We show that by using an inoculum at an optical density at 530 nm (OD_{530}) of 0.11, *A. fumigatus* AMB and ITC MIC_{RSA} s obtained at 16 h were equal to or within a single twofold dilution of AMB and ITC MIC_{NCCLS} s obtained at 48 h. Preliminary testing with *Aspergillus terreus* also showed that by using an 0.11- OD_{530} inoculum, AMB and ITC MIC_{RSA} s obtained at 16 h were equal to or within a single twofold dilution of AMB and ITC MIC_{NCCLS} s obtained at 48 h.

MATERIALS AND METHODS

Reagents. Preparations of RPMI 1640 without glucose (catalog no. R-1383; Sigma, St. Louis, Mo.) or with 0.4% glucose were buffered with 0.165 M 3-(N-morpholino)-propanesulfonic acid (catalog no. 19256; U.S. Biochemicals, Cleveland, Ohio) and adjusted to pH 7.0 with 10 N NaOH.

Fungal Strains. *A. fumigatus* strains NCPF 7102, 7101, 7100, 7098, and 7097 were purchased from the National Collection of Pathogenic Fungi (Mycology Reference Laboratory, Bristol, United Kingdom). These strains were isolated from aspergillosis patients with known clinical outcomes. *A. terreus* strain 55 was donated from the University of Virginia Health System. Species designations of *A. fumigatus* and *A. terreus* strains were confirmed by observations of macroscopic and microscopic characteristics during growth on Czapek-Dox agar (9). All stocks were maintained in vials of sterile water at 4°C.

Antifungal agents and dilutions. AMB (A-9528; Sigma) and ITC (catalog no. R51211; Research Diagnostics Inc., Flanders, N.J.) were purchased in powdered form. Stocks of 3,200 μ g/ml were prepared in dimethyl sulfoxide (DMSO) (D-

* Corresponding author. Mailing address: Research Institute for Children, 200 Henry Clay Ave., New Orleans, LA 70118. Phone: (504) 896-2712. E-mail: twetter@chnola-research.org.

2650; Sigma) and stored at -20°C for up to 6 months. For both RSA and NCCLS M38-P testing, serial twofold dilutions were prepared following NCCLS M38-P guidelines (13), except that the final $2\times$ concentration range of 0.06 to 32 μg of AMB or ITC/ml was prepared in 0.4% glucose RPMI 1640. Both drug-free growth and glucose controls were also prepared, containing the same ratio of DMSO to 0.4% RPMI 1640 as the $2\times$ drug dilutions.

Microwell plate preparation. Each NCCLS M38-P and RSA test was performed in duplicate in 96-well round-bottom plates (catalog no. 3799; Costar, Corning, N.Y.), with each row, comprised of 12 wells, corresponding to one susceptibility test. All testing was performed at least three times on separate days. Wells 1 to 10 were inoculated with either 100 μl of the $2\times$ AMB or ITC dilution range (0.06 to 32 $\mu\text{g}/\text{ml}$). Wells 11 and 12, the drug-free growth control and glucose control, respectively, were inoculated with 100 μl of 0.4% glucose-DMSO RPMI 1640. Plates were prepared on the day of use and stored at 4°C until needed.

Inoculum preparation. Isolates were cultured on Sabouraud glucose agar (catalog no. 211584; BBL, Cockeysville, Md.) slants at 30°C for 3 days (*A. fumigatus*) or 7 days (*A. terreus*). After flooding with glucose-deficient RPMI 1640, the slant surface was gently scraped with a sterile wooden applicator stick. After the heavier hyphal fragments had settled, approximately 15 min, the conidial suspension was collected and adjusted with glucose-deficient RPMI 1640 to an OD_{530} of 0.46, 0.26, or 0.11. The 0.11- OD_{530} conidial preparation is referred to as the RSA density conidial inoculum (CI_{RSA}).

NCCLS M38-P recommended conidial inocula (13) used in NCCLS M38-P and RSA testing were prepared by diluting the CI_{RSA} 1:50 with glucose-deficient RPMI. The 1:50-diluted conidial preparation is referred to as the NCCLS density conidial inoculum (CI_{NCCLS}). All conidial inocula were kept on ice after preparation and used within 3 h.

RSA. The RSA was initiated by inoculating wells 1 to 11 with 100 μl of CI_{RSA} or CI_{NCCLS} and well 12 with 100 μl of glucose-deficient RPMI and incubating at 35 to 37°C . Residual glucose levels were detected by addition of 50 μl of an enzyme color mix comprised of 0.6 M sodium phosphate buffer (pH 6.0), 360 μg of 4-amino antipyrine (A4382; Sigma)/ml, 490 μg of *N*-ethyl-*N*-sulfonylpropyl-*m*-toluidine (E8506; Sigma)/ml, 0.68 U of horseradish peroxidase (P8415; Sigma)/ml, and 0.4 U of glucose oxidase (catalog no. 195196; ICN Biochemicals, Aurora, Ohio)/ml to each well. At 20 min, the color intensity was measured at OD_{550} with a reference reading of OD_{655} in a plate reader (Benchmark Plus; Bio-Rad, Hercules, Calif.).

RSA antifungal susceptibility curves and MIC endpoint determination. The percent residual glucose of each test well was calculated by comparing the OD_{550} of the test well with the OD_{550} of the glucose control well based on the following equation: $(\text{OD}_{550} \text{ of test well} / \text{OD}_{550} \text{ of glucose control well}) \times 100$. The percent inhibition of glucose utilization due to AMB or ITC was determined by the following equation: $1 - [(100\% - \% \text{ residual glucose of growth control}) / (100\% - \% \text{ residual glucose of growth control})] \times 100$. Antifungal susceptibility curves were generated for each RSA by plotting the percent residual glucose values (y axis) against the appropriate AMB or ITC concentrations (x axis). The resulting detection interval contained an upper and lower limit represented, respectively, by the highest concentration of antifungal drug and the drug-free growth control. AMB MIC_{RSA} endpoint determinations were defined as the lowest drug concentration having a percent residual glucose value within the top 10% of the detection interval. The ITC MIC_{RSA} endpoint was defined as the lowest drug concentration corresponding to a residual glucose value within the top 20% of the detection only if (i) a detection interval comprised of an upper plateau, steep decline, and lower plateau was discernible and (ii) the highest concentration of ITC (16 $\mu\text{g}/\text{ml}$) had a corresponding residual glucose value of $\geq 80\%$. If these conditions were not met, the ITC MIC_{RSA} was designated as >16 $\mu\text{g}/\text{ml}$.

M38-P testing and MIC endpoint determination. The NCCLS M38-P assay was initiated by inoculating wells 1 to 11 with 100 μl of CI_{NCCLS} and well 12 with 100 μl of glucose-deficient RPMI and incubation at 35 to 37°C . Although the AMB and ITC dilutions were prepared in 0.4% glucose RPMI and the conidial inocula were prepared in glucose-deficient RPMI, the final concentration of glucose was 0.2% at the time of incubation in accordance with M38-P procedures. $\text{MIC}_{\text{NCCLS}}$ s were determined by visual inspection at 48 h using a mirror plate reader (Cooke Engineering Co., Alexandria, Va.) according to NCCLS M38-P guidelines (13) with AMB $\text{MIC}_{\text{NCCLS}}$ endpoints designated as the lowest AMB concentration preventing growth and ITC $\text{MIC}_{\text{NCCLS}}$ endpoints designated as the lowest ITC concentration reducing growth by 50% compared to the growth control.

Statistics. Analysis of variance was used for comparison of residual glucose values determined after conidial inocula incubations with 16 μg of AMB or ITC/ml for 24 h. A *P* value of <0.05 was considered significant.

FIG. 1. Conidial development during 24-h incubation at 37°C . Wet-mount slide preps were observed ($\times 400$) at 3 h (A), 6 h (B), 8 h (C), and 24 h (D) with no antifungal agent; 24 h with 16 μg of AMB/ml (E); and 24 h with 16 μg of ITC/ml (F).

RESULTS

Conidial growth and glucose utilization. To determine the relationship of conidial development to glucose utilization in the absence of antifungal agents, CI_{RSA} were prepared from *A. fumigatus* NCPF strains 7102, 7101, 7100, 7098, and 7097 and incubated in 0.2% glucose RPMI at 37°C for 24 h. Observations of wet mounts prepared at 0, 3, 6, 8, and 24 h showed that conidia of all strains initiated swelling, germination, and hyphal extension at similar time points (data not shown). Conidial morphology remained unaltered (Fig. 1A) for the first few hours, with conidial swelling becoming clearly evident at 6 h (Fig. 1B). By 8 h, germ tubes emerged (Fig. 1C) and progressed to increasingly more complex hyphal development throughout the remainder of the 24-h incubation (Fig. 1D). The pattern of uninhibited glucose utilization was similar between strains during the 24-h incubation (Fig. 2). Rates of glucose utilization were greatest between 0 and 16 h, with residual glucose levels decreasing from 100% to 40 to 50% at 16 h. Decreased rates of glucose utilization were observed between 16 and 24 h, with final residual glucose values of 30 to 40%.

FIG. 2. Glucose utilization by *A. fumigatus* strains during 24-h incubation at 37°C. NCPF strains included 7102 (○), 7101 (●), 7100 (■); 7098 (▽); and 7097 (▼).

Antifungal effect on conidial development and glucose utilization. To determine the effect of AMB and ITC on conidial development and glucose utilization, CI_{RSA} were prepared with all *A. fumigatus* strains and incubated in 0.2% glucose RPMI containing 16 μ g of AMB or ITC/ml at 37°C for 24 h. AMB had a similar effect on all strains, with inhibition of conidial development occurring prior to swelling (Fig. 1E) and residual glucose values of 94.3% at 24 h, corresponding to 90% inhibition of glucose utilization (Fig. 3). With the exception of strain NCPF 7100, ITC inhibited conidial development at a partially swollen state (Fig. 1F), with residual glucose values of 91.4% at 24 h, corresponding to 85% inhibition of glucose utilization (Fig. 3A). The ITC concentration of 16 μ g/ml was noninhibitory for strain NCPF 7100, as shown by hyphal development (data not shown) and a residual glucose value of 65%, i.e., 22% glucose inhibition, at 24 h (Fig. 3B).

Optimization of rapid susceptibility assay conditions. To determine the optimal RSA conditions of incubation time and conidial inoculum, AMB and ITC RSAs were performed with all strains at 16, 24, and 48 h using CI_{RSA} and CI_{NCCLS} . Op-

timal incubation time and conidial inoculum density conditions were defined as those providing a MIC_{RSA} equal to or within a single twofold dilution of a MIC_{NCCLS} within the shortest period of incubation. Preliminary testing with conidial inocula of OD_{530} s of 0.26 and 0.44 provided unsatisfactory results (data not shown) and was not used in optimization experiments. The AMB RSA susceptibility curves for the CI_{RSA} and CI_{NCCLS} of strains NCPF 7101 and 7100 at 16 h were characterized by a detection interval composed of an upper plateau representing inhibitory concentrations of AMB, a steep decline representing the dose-dependent inhibitory range, and a lower plateau representing noninhibitory concentrations of AMB (Fig. 4). While residual glucose values in the upper plateau remained fairly constant with increased incubation periods, residual glucose values representing the lower plateau decreased with increased incubation periods.

The AMB MIC_{RSA} endpoint was defined as the lowest AMB concentration corresponding to a residual glucose value in the upper 10% of the detection interval. For all strains tested, AMB MIC_{RSA} s obtained at 16 h were equal to or within a single twofold dilution of MIC_{RSA} s obtained at 48 h regardless of conidial inoculum density (Table 1). More importantly, AMB MIC_{RSA} s obtained with conidial inocula of either density at 16 h were equal to or within a single twofold dilution of MIC_{NCCLS} s obtained at 48 h.

With the exception of strain NCPF 7100, ITC RSA susceptibility curves (Fig. 5A and B) of all strains were similar to AMB susceptibility curves, with a detection interval comprised of a drug-sensitive upper plateau, dose-dependent decline in sensitivity, and drug-insensitive lower plateau. ITC MIC_{RSA} endpoints were defined as the lowest ITC concentration corresponding to a residual glucose value in the upper 20% of the detection interval. As with AMB, ITC MIC_{RSA} s at 16 h were equal to or within a single twofold dilution of MIC_{RSA} s and MIC_{NCCLS} s obtained at 48 h (Table 1).

Strain NCPF 7100 showed differences between susceptibility curves for the two inoculum densities tested. The CI_{RSA} sus-

A. Strains NCPF 7102, 7101, 7098, 7097

B. Strain NCPF 7100

FIG. 3. Glucose utilization by *A. fumigatus* strains during 24-h incubation at 37°C in the presence of no antifungal agent, 16 μ g of AMB/ml, or 16 μ g of ITC/ml. (A) Average glucose utilization of strains NCPF 7102, 7101, 7098, and 7097 in the presence of no antifungal drug (●), 16 μ g of AMB/ml (○), or 16 μ g of ITC/ml (▼). (B) Average glucose utilization of strain NCPF 7100 in the presence of no antifungal drug (●), 16 μ g of AMB/ml (○), or 16 μ g of ITC/ml (▼).

FIG. 4. AMB susceptibility curves for strains 7101 and 7100 at 16 h (●), 24 h (○), and 48 h (▼) in the presence of 0.03 to 16 μ g of AMB/ml. CI_{RSA} results are shown in panels A and C, while CI_{NCCLS} results are shown in panels B and D. MICs in each curve are designated with a box. These data represent one experiment. This experiment was performed three times, resulting in similar curves with MIC points equal to or within a single twofold dilution of the MIC points shown. *A. fumigatus* strains NCPF 7102, 7098, and 7097 gave results similar to those for NCPF 7101 and 7100.

ceptibility curves had little or no detection intervals, with residual glucose values of $\leq 72\%$ corresponding to 16 μ g of ITC/ml at 16, 24, and 48 h (Fig. 5C). The 16-h CI_{NCCLS} susceptibility curve had a well-defined detection interval, with a residual glucose value of 91% corresponding to 16 μ g/ml. However, at 24 and 48 h, detection intervals were indiscernible, with residual glucose values of 66 and 60% corresponding to 16 μ g of ITC/ml, respectively (Fig. 5D). The CI_{RSA} ITC MIC_{RSA} of >16 μ g/ml at 16, 24, and 48 h was equal to the ITC MIC_{NCCLS} determined at 48 h (Table 1). The CI_{NCCLS} ITC MIC_{RSA} was 1 μ g/ml at 16 h but increased to >16 μ g/ml by 24 h.

***A. terreus* RSA testing.** Preliminary AMB and ITC RSA testing was performed with CI_{RSA} and CI_{NCCLS} of *A. terreus* strain 55 at 16, 24, and 48 h. Use of a CI_{RSA} provided AMB and ITC MIC_{RSA} s at 16 h equal to or within a single twofold dilution of AMB and ITC MIC_{NCCLS} s obtained at 48 h, while a CI_{NCCLS} required 24 h for AMB and ITC MIC_{RSA} determination (Table 1).

DISCUSSION

The principle objective of this study was to modify the yeast RSA to facilitate antifungal susceptibility testing of *A. fumigatus*. To convert the yeast RSA to a mold RSA, the reliability of residual glucose detection as an indicator of antifungal effect on conidial development was first investigated. Conidia of all strains were similar in that the greatest glucose utilization occurred during conidial swelling and germination (0 to 16 h), with less utilization after hyphae had developed (16 to 24 h). When conidia were incubated in the presence of 16 μ g of AMB or ITC/ml, AMB caused rapid inhibition prior to 6 h, since conidia did not develop past the preswollen state. ITC inhibited conidial development at a later phase than AMB but prior to 6 h, since conidia were inhibited in a partially swollen state. Because AMB interacts directly with membrane ergosterol (1), whereas ITC inhibits ergosterol synthesis (10, 16), the differential inhibitory effect of the two drugs was not unexpected. This difference was also consistent with inhibition of conidial glucose utilization in 16 μ g of either drug/ml. AMB inhibited

TABLE 1. RSA (CI_{RSA} and CI_{NCCLS}) and NCCLS M38-P MICs at 16, 24, and 48 h^a

<i>Aspergillus</i> sp.	NCPF no.	Incubation period (h)	AMB MIC (μ g/ml)			ITC MIC (μ g/ml)		
			RSA		NCCLS	RSA		NCCLS
			CI_{RSA}	CI_{NCCLS}	CI_{NCCLS}	CI_{RSA}	CI_{NCCLS}	CI_{NCCLS}
<i>A. fumigatus</i>	7100	16	1	0.5		>16	1	
		24	1	1		>16	>16 ^b	
		48	2	1	1	>16	>16	>16
	7098	16	0.5	0.25		0.5	0.5	
		24	0.5	0.5		1	0.5	
		48	1	0.5	0.5	1	0.5	0.5
	7097	16	0.5	0.5		0.5	0.5	
		24	0.5	0.5		0.5	0.5	
		48	1	0.5	0.5	0.5	0.25	0.25
	7102	16	0.5	0.5		0.5	0.25	
		24	0.5	0.5		0.25	0.5	
		48	1	0.5	0.5	0.5	0.5	0.25
	7101	16	0.5	0.25		1	0.25	
		24	0.5	0.5		1	0.5	
		48	1	0.5	0.5	1	0.5	0.5
<i>A. terreus</i>		16	0.5	≤ 0.03		0.25	≤ 0.03	
		24	1	0.5		0.25	0.125	
		48	4	1	1	0.25	0.25	0.25

^a These data represent a single experiment. The experiment was repeated two additional times with MICs equal to or within a single twofold dilution of results shown.

^b Variable MIC results (1 to 16 μ g/ml).

90% of the glucose utilization (94.3% residual glucose) over a 24-h incubation period, while ITC inhibited 85% (91.4% residual glucose). Since these residual glucose values were marginally, but not significantly, different ($P > 0.5$), we concluded that ITC inhibited conidial development only a short period after AMB. Since AMB and ITC inhibited conidial development in a nonswollen and partially swollen state prior to 6 h, respectively, the correlation between inhibition of growth and inhibition of glucose utilization could be demonstrated only if the corresponding residual glucose values at the times of AMB and ITC inhibition were similar to those shown in the drug-free control prior to 6 h. These residual glucose values of 94.3 and 91.4%, corresponding to 16 μ g of AMB and ITC/ml, respectively, fall within the residual glucose value range of 100 to 84% between 0 and 6 h in the drug-free control. These data indicated that inhibition of growth, the criterion used in current susceptibility testing, such as the NCCLS M38-P assay, is correlated with inhibition of glucose utilization, and glucose utilization can thus be used as an indicator of conidial susceptibility to AMB and ITC.

Factors of the yeast RSA requiring modification for the mold RSA were inoculum concentration, incubation time, and MIC endpoint determination. Optimal conditions produced susceptibility curves composed of a clearly defined upper plateau (inhibitory doses of drug), a steep decline (dose-dependent inhibition), and a lower plateau (noninhibitory doses), with few or no residual values in the dose-dependent decline. Preliminary mold RSA experiments with conidial inocula with an OD_{530} of 0.11, 0.26, and 0.42 showed that as the conidial concentration increased from an OD_{530} of 0.11 to OD_{530} s of 0.26 and 0.42, the susceptibility curves became less defined, with a dose-dependent decline of decreased slope containing increasing numbers of values (data not shown). Since our results showed that the optimal CI_{RSA} is a suspension of conidia with a turbidity equivalent to an OD_{530} of 0.9 to 0.11, whereas

the NCCLS M38-P assay defines the inoculum as a 1:50 dilution of that turbidity (CI_{NCCLS}), we compared RSA MICs obtained for each inoculum concentration. Analysis of AMB susceptibility curves (Fig. 4) for both inocula led to several generalizations regarding AMB detection intervals: (i) susceptibility curves are well defined at each time interval regardless of inoculum density; (ii) all strains produced similar susceptibility curves at each incubation period, with longer incubation periods resulting in broader detection intervals; and (iii) inhibitory concentrations of AMB (located in the upper plateau) at 48 h correspond to residual glucose values in the top 10% of the detection interval at 16 h, regardless of inoculum density. Based on this last observation, the AMB MIC_{RSA} endpoint was defined as the lowest AMB concentration having a percent residual glucose value in the upper 10% of the detection interval. According to this criterion, either inoculum density can be used to obtain AMB MIC_{RSA}s at 16 h.

Analysis of ITC susceptibility curves (Fig. 5) for all strains except NCPF 7100 resulted in conclusions similar to those reached for the AMB MIC_{RSA} above except that inhibitory concentrations of ITC at 48 h corresponded with percent residual glucose values in the top 20% (>83% residual glucose), rather than the top 10%, of the detection interval at 16 h, thus defining the ITC MIC_{RSA} endpoint as the lowest ITC concentration corresponding to a percent residual glucose value in the upper 20% of the detection interval. This criterion could be used for MIC determination only if two other requirements were also met: (i) the presence of a clearly discernible susceptibility curve composed of an upper plateau, drug-sensitive decline, and lower plateau and (ii) residual glucose values of $\geq 80\%$ in the upper plateau. If these conditions were not met, the MIC was designated at >16 μ g/ml. These additional requirements were established based on comparisons of the 16-h CI_{RSA} susceptibility curves of strains NCPF 7100 and 7101 (Fig. 5A and C). The NCPF 7100 susceptibility curve had a

FIG. 5. ITC susceptibility curves for strains 7101 and 7100 at 16 h (●), 24 h (○), and 48 h (▼) in the presence of 0.03 to 16 μ g of ITC/ml. CI_{RSA} results are shown in panels A and C, while CI_{NCCLS} results are shown in panels B and D. MICs in each curve are designated with a box if the value is ≤ 16 μ g/ml. These data represent one experiment. This experiment was performed three times, resulting in similar curves with MIC points equal to or within a single twofold dilution of the MIC points shown (except CI_{NCCLS} of 7100 at 24 h). *A. fumigatus* strains NCPF 7102, 7098, and 7097 gave results similar to those for NCPF 7101.

slope in the dose-dependent decline approaching zero, since all ITC residual glucose values were nearly the same as those for the drug-free control, with a 72% residual glucose value corresponding to 16 μ g of ITC/ml (Fig. 5C). In contrast, strain NCPF 7101 had a defined susceptibility curve, with residual glucose values of $>80\%$ in the upper plateau (Fig. 5A). Since strain NCPF 7100 lacked both a discernible susceptibility curve and residual glucose values of $\geq 80\%$, its MIC_{RSA} was designated as >16 μ g/ml. Comparison of 16-h CI_{RSA} and CI_{NCCLS} susceptibility curves also showed that a CI_{RSA} is superior to the CI_{NCCLS} , since the latter produced an erroneous ITC MIC_{RSA} of 1 μ g/ml at 16 h.

Denning et al. used strains NCPF 7102, 7101, 7100, 7098, and 7097 in agar- and broth-based susceptibility testing studies (6, 8). Strain NCPF 7100 was obtained from a patient who did not respond to ITC therapy; thus, this strain was deemed in vivo resistant to ITC, whereas NCPF 7098 was obtained from a patient who responded to ITC and was deemed in vivo susceptible. Importantly, these susceptibility conclusions were supported by in vitro MIC_{NCCLS} s, suggesting that in vitro MIC

testing may have predictive value of therapeutic outcome for aspergillosis patients treated with ITC. In contrast, MIC results for isolates from aspergillosis patients with failed therapy, strains NCPF 7101 and 7097, led Denning et al. to conclude that in vivo resistance to AMB does not correlate with in vitro MIC_{NCCLS} results.

ITC and AMB MIC_{RSA} s obtained in our studies have led us to identical conclusions. The CI_{RSA} ITC MIC_{RSA} of >16 μ g/ml for strain NCPF 7100 at 16 h correlates with the clinical history of in vivo ITC resistance. In vivo resistance to AMB did not correlate with in vitro resistance, since testing of NCPF 7102 and 7097 resulted in AMB MIC_{RSA} s of 0.5 and 1 μ g/ml at 16 h, respectively, indicating in vitro drug susceptibility.

Results of studies with strains NCPF 7102 and 7097 with experimental animals (6, 8) suggest that host factors may explain why in vitro AMB MICs do not necessarily correlate with in vivo outcome. For example, NCPF 7102, which was deemed in vivo resistant based on patient outcome, was responsive to AMB therapy in experimental animals (6, 8). Clearly, further

work needs to be done to determine whether host status may be taken into account when interpreting AMB MIC results.

Preliminary testing was performed with *A. terreus* to assess whether the RSA could be used with medically significant species other than *A. fumigatus*. As with *A. fumigatus*, only a CI_{RSA} provided AMB and ITC MIC_{RSA} s at 16 h that were equal to or within a single twofold dilution of AMB and ITC MIC_{NCCLS} s. While the ITC MIC_{RSA} remained unchanged between 16 and 48 h of incubation, it was interesting that the AMB MIC_{RSA} increased from 0.5 μ g/ml at 16 h to 4 μ g/ml at 48 h. Since we did not observe eightfold MIC_{RSA} increases with *A. fumigatus* isolates between 16 and 48 h, we hypothesize that this phenomenon may be due to the slower growth rate of *A. terreus* compared to that of *A. fumigatus*. The fourfold difference in the CI_{RSA} , MIC_{RSA} and MIC_{NCCLS} s, 4 and 1 μ g/ml, respectively, at 48 h was also an unexpected result. One possible explanation for this is that the CI_{RSA} , 50 times greater than the CI_{NCCLS} , produced a skewed AMB MIC_{RSA} . This, however, seems unlikely, since use of a CI_{RSA} resulted in ITC MIC_{RSA} s at 16, 24, and 48 h that were equal to the MIC_{NCCLS} . Another possibility is that the RSA provides more accurate MICs than the NCCLS M-38P assay. Numerous studies have suggested that *A. terreus* is naturally resistant to AMB therapy in vivo (3, 7), which is more consistent with the 4- μ g/ml AMB MIC_{RSA} than the AMB MIC_{NCCLS} of 1 μ g/ml. The testing of additional *A. terreus* strains with detailed clinical histories and/or animal experimentation data may well help resolve this issue.

In conclusion, these data show that the detection of glucose utilization can be used to predict the in vitro susceptibility of *A. fumigatus* to AMB and ITC at 16 h of incubation. Preliminary RSA testing with the slower-growing *A. terreus* also suggests that the ITC and AMB MIC_{RSA} can be determined by 16 h. This assay is an improvement over the current susceptibility assay, since MIC_{RSA} s are quantitative and objective, while the MIC_{NCCLS} s are subjective and rely on observation of growth. Furthermore, the RSA is relatively inexpensive and requires

less effort, and more information can be obtained than with NCCLS M38-P testing.

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