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Comparison of the Semisolid Agar Antifungal Susceptibility Test with the NCCLS M38-P Broth Microdilution Test for Screening of Filamentous Fungi

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Antifungal susceptibility testing of pathogenic molds is being developed. A simple screening semisolid agar antifungal susceptibility (SAAS) test accurately measures susceptibilities of yeasts. The performance of the SAAS screening test for filamentous fungi was assessed by comparing MICs of four antifungals (amphotericin B [AMB], AMB lipid complex [ABEL], itraconazole [ITZ], and posaconazole [POS]) for 54 clinical mold isolates with the results of the National Committee for Clinical Laboratory Standards (NCCLS) proposed broth microdilution method (M38-P). The SAAS test utilized inocula stabbed into tubes of 0.5% semisolid heart infusion agar. In both tests MICs were read after incubation at 35°C for 48 h. The isolates tested were *Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus flavus*, other *Aspergillus* spp., *Fusarium* spp., *Penicillium* sp., *Mucor* sp., *Scedosporium prolificans*, *Trichophyton* sp., and an unidentified dematiaceous mold. Concordance of test results was determined as the percent agreement of MICs ± 1 dilution. The overall agreement between the tests for each drug was as follows: AMB, 94%; ABEL, 83%; ITZ, 94%; POS, 94%. For the *Aspergillus* spp., all but one were susceptible to ITZ by SAAS test; all were susceptible to POS (MIC range, 0.25 to 4 μg/ml). Three of six non-*Aspergillus* molds that were resistant to AMB and ABEL by SAAS (MIC ≥ 2 μg/ml) were also resistant by the NCCLS test. The SAAS test compared favorably to the NCCLS broth microdilution test for molds, and most of the clinical isolates tested were susceptible to all four drugs.

Invasive mold infections occurring primarily in immunocompromised hosts are associated with significant morbidity and mortality despite advances in antifungal therapy in the past decade (6, 7). Although treatment options are now expanded, emerging pathogens such as *Fusarium* spp., *Scedosporium prolificans*, and *Aspergillus terreus* may be resistant to the currently available antifungal agents. Predicting antifungal drug resistance of an organism to available drugs is the goal of antifungal susceptibility testing, which, in turn, might aid in timely and successful intervention in these life-threatening infections (11).

Antifungal susceptibility testing of molds is being developed. The NCCLS has developed a standardized method of broth testing utilizing nongerminated conidial inoculum suspensions (8). The test employs a methodology similar to that for yeasts but requires spectrophotometric inoculum determination based on conidial size. Interlaboratory agreement is high for the broth dilution test, thus making it suitable as a reference standard (1, 2). However, it is somewhat cumbersome to perform and not likely to be used in clinical microbiology laboratories (3).

The semisolid agar antifungal susceptibility (SAAS) screening test has performed well when compared with NCCLS broth microdilution testing for yeasts and might be a useful preliminary screening test for molds (9). This test for filamentous fungi uses inocula prepared from a colony swab, without the need for special equipment. Suspensions of mostly conidia made from 3-day-old cultures result in final inocula of approximately 0.2×10^2 CFU/ml. Filamentous growth in the test tube of semisolid agar can be visualized and scored as 0 (100% inhibition), 1+ (≥75% inhibition), 2+ (~50% inhibition), 3+ (~25% inhibition), or 4+ (growth equal to that of control) when various drug dilutions are used.

The purpose of this study was to compare results of antifungal susceptibility testing of more than 50 clinical mold isolates from a single institution utilizing the then-available NCCLS broth microdilution and the SAAS methods against four antifungal agents: itraconazole (ITZ); posaconazole (POS); amphotericin B (AMB); and a lipid formulation of AMB, AMB lipid complex (ABEL). Concordance of results by each method plus or minus 1 dilution was assessed.

MATERIALS AND METHODS

Test isolates. Fifty-four strains of filamentous fungi obtained from clinical specimens were studied. These included *Aspergillus fumigatus* ($n = 30$); *Aspergillus niger* ($n = 9$); *Aspergillus* spp. ($n = 5$); *Aspergillus flavus* ($n = 3$); *Fusarium* spp. ($n = 2$); and one each of *Trichophyton* sp., *Penicillium* sp., *Mucor* sp., *S. prolificans*, and a dematiaceous mold. *Candida krusei* ATCC 6258 was used as a quality control for each test.

Antifungal agents. Four antifungal agents were studied. These included conventional AMB (Calbiochem, San Diego, Calif.), used for the NCCLS test; AMB in the form of Fungizone (Apothecon Division of Bristol Myers Squibb, Princeton, N.J.), used for the SAAS test; and for both tests, AMB lipid complex (kindly provided by The Liposome Company, Princeton, N.J.), ITZ (kindly provided by Janssen Pharmaceutica, Beerse, Belgium), and POS (kindly provided by Schering Plough Research Institute, Kenilworth, N.J.). Each drug was prepared according to the manufacturer's directions and as described for the NCCLS method.

SAAS test: medium, drug dilutions, and inoculum preparation. Five-ml aliquots of a single lot of heart infusion (HI) broth (Difco Laboratories, Detroit, Mich.) containing 0.5% agar (Bacto Agar; Difco Laboratories) at a pH of ~7.4 were prepared in glass tubes (16 by 125 mm) under sterile conditions.

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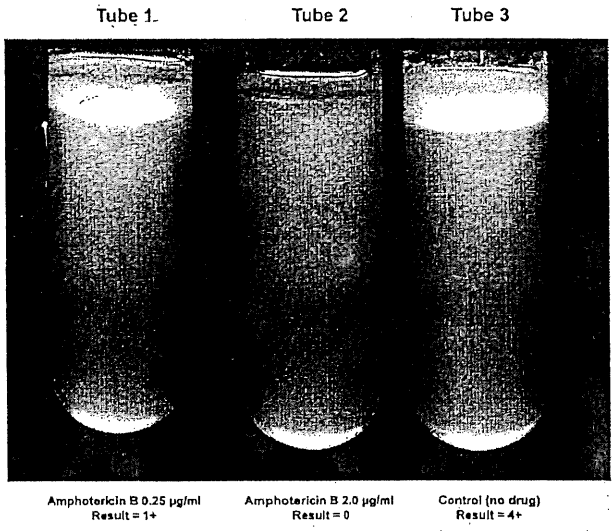


FIG. 1. Example of endpoint determination for filamentous fungi by SAAS. *A. fumigatus* exposed to 0.5% semisolid HI agar containing AMB at 0.25 µg/ml (left, tube 1), AMB at 2 µg/ml (middle, tube 2), and no drug (right, tube 3) after 48 h of incubation at 35°C. Tube 1 represents 1+ growth; tube 2 represents 0 growth (complete inhibition); tube 3 represents 4+ growth (equal to control). The filamentous growth is detected within the agar when visually inspected.

Stock solutions of drugs were made as follows. AMB and ABEL were dissolved in sterile water at 5,000 µg/ml; ITZ and POS were dissolved in 100 and 10% dimethyl sulfoxide, respectively, at 1,600 µg/ml. The same diluents were used to make twofold dilutions. The diluted drugs were added to 5-ml aliquots of molten 0.5% HI agar kept at 45 to 50°C to achieve the following range of final concentrations: for AMB and ABEL, 0.125 to 2.0 µg/ml; for ITZ, 0.25 to 2 µg/ml; and for POS, 0.25 to 8 µg/ml.

Fungi were grown for 7 days at 35°C on Sabouraud dextrose agar slants. After adding 1 drop of Tween 20, the colonies were brushed with a sterile cotton swab and the mixture of conidia and hyphal fragments was suspended in 1 ml of sterile water. Heavy particles were allowed to settle after vortexing, and the homogenous suspension was adjusted to achieve a turbidity of ~0.5 McFarland standard. A standard platinum loopful (0.001 ml) of the inoculum suspension was inserted into each 5-ml aliquot of semisolid HI broth containing the indicated drugs or a drug-free control. A loopful of the inoculum suspension was also streaked onto Sabouraud dextrose agar to check for purity and viability. The tubes were overlaid with 0.5 ml of sterile mineral oil to inhibit sporulation and incubated at 35°C for 48 h.

NCCLS test. Drug preparations and dilutions for the NCCLS method were performed in accordance with the NCCLS M38-P document, which was the only version of the document available at the time. Final concentrations for all drugs ranged from 0.03 to 16 µg/ml. RPMI 1640 medium (Gibco BRL, Grand Island, N.Y.) and antibiotic medium 3 (AM3) (Difco) were used for the NCCLS test, and inocula were prepared as per protocol (8, 9). Plates were incubated at 35°C for 48 h.

Endpoint determination. For both the SAAS and NCCLS tests, growth in all tubes or wells was compared to that of drug-free control and scored by visual inspection as follows: 4+, growth comparable to control; 3+, growth 75% of control; 2+, growth 50% of control; 1+, growth 25% of control; and 0, no growth. Figure 1 depicts endpoints of growth of an *A. fumigatus* isolate in the semisolid agar HI medium with AMB (0.25 and 2.0 µg/ml) and with no drug (tubes 1, 2, and 3, respectively).

The MIC of the antifungal agent was determined according to the class of drug as per the NCCLS guidelines at the time (8). MICs of the fungicidal agents AMB and ABEL were defined as the lowest concentration at which there was complete growth inhibition. MICs of ITZ and POS were defined as the lowest concentration at which there was 50% growth inhibition (i.e., a score of ≤2+).

Comparison of tests. Concordance of MIC results between tests was defined as the percentage of MIC results by both methods that fell within 3 drug dilutions (±1 dilution) for each isolate.

RESULTS AND DISCUSSION

The SAAS and NCCLS MIC range, MIC at which 50% of strains were inhibited (MIC_{50}), and MIC_{90} for each drug and medium tested against the *Aspergillus* spp. are presented in Table 1. The majority of these clinical isolates were susceptible to all four drugs. Table 2 presents the results of susceptibility testing of the non-*Aspergillus* isolates tested. The one *S. prolificans* blood isolate demonstrated reduced susceptibility to all four drugs by both NCCLS and SAAS methods (Table 2). The

TABLE 1. MIC results for *Aspergillus* species by test and medium

Organism and medium[a]	MIC[b] (µg/ml)											
	AMB			ABEL			ITZ			POS		
	Range	50%	90%	Range	50%	90%	Range	50%	90%	Range	50%	90%
A. fumigatus (n = 30)												
SAAS	0.125–2	0.5	1	0.125–>2	0.25	1	0.25–>2	0.25	0.25	0.25–4	0.25	0.25
NCCLS-RPMI	0.25–1	0.5	1	0.03–0.5	0.125	0.25	0.06–16	0.125	0.5	0.06–16	0.125	0.125
NCCLS-AM3	0.125–4	0.25	0.5	0.03–16	0.125	0.25	0.06–16	0.125	0.25	0.03–16	0.06	0.125
A. niger (n = 9)												
SAAS	0.125–1	0.5	0.5	0.125–0.5	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
NCCLS-RPMI	0.25–0.5	0.25	0.5	0.06–0.5	0.125	0.5	0.06–0.5	0.25	0.5	0.03–0.25	0.125	0.25
NCCLS-AM3	0.125–0.5	0.25	0.5	0.03–0.25	0.06	0.25	0.03–0.5	0.25	0.5	0.03–0.125	0.06	0.125
Aspergillus spp. (n = 5)												
SAAS	1–2			0.5–2			0.25			0.25		
NCCLS-RPMI	0.125–1			0.06–0.25			0.03–0.125			0.03–0.125		
NCCLS-AM3	0.5–2			0.125–1			0.03–0.125			0.03–0.125		
A. flavus (n = 3)												
SAAS	0.25–1			0.125–1			0.03–0.125			0.03–0.125		
NCCLS-RPMI	0.125–1			0.06–0.125			0.06–0.125			0.125		
NCCLS-AM3	0.25–1			0.06–0.125			0.06–0.125			0.03–0.06		

[a] Abbreviations: NCCLS, National Committee for Clinical Laboratory Standards; RPMI, RPMI 1640 medium; AM3, antibiotic 3 medium.
[b] 50 and 90%, MIC_{50} and MIC_{90}, respectively.

TABLE 2. MICs of single isolates of non-*Aspergillus* filamentous fungi as determined by SAAS and NCCLS tests

Isolate	MIC (μg/ml) as determined by test							
	SAAS				NCCLS RPMI			
	AMB	ABEL	ITZ	POS	AMB	ABEL	ITZ	POS
Dematiaceous	1	0.25	0.25	0.25	1	0.125	0.125	0.125
Fusarium sp.	0.5	>2	2	2	1	0.25	16	0.5
Fusarium sp.	0.5	2	0.25	0.25	16	>16	>2	>16
Mucor sp.	<0.25	0.25	>2	>8	0.25	0.5	>16	>16
Penicillium sp.	1	0.25	>2	2	0.5	0.06	16	16
Trichophyton sp.	1	0.25	0.25	0.25	1	0.125	0.25	0.125
S. prolificans	>2	>2	>2	>8	>16	>16	>16	>16

two *Fusarium* isolates differed the most in MICs by the two methods. Testing was repeated four times for the first isolate and yielded similar results. The SAAS test predicted POS susceptibility for both isolates, whereas the POS MIC by NCCLS was >16 for the second isolate.

The concordance of MIC results by SAAS and NCCLS within 1 dilution is depicted in Table 3. There appears to be less concordance comparing methods for ABEL than is the case with the three other drugs. However, most MICs documented in our study by the NCCLS method were less than 0.25 μg/ml and therefore below the lowest MIC detectable by the SAAS method due to the design of the study. In contrast, MICs for two *Aspergillus* sp. isolates measured by the SAAS method predicted reduced susceptibility (high MIC) to both AMB preparations compared with results obtained by NCCLS testing.

The lowest azole concentration tested by SAAS was 0.25 μg/ml. All but five clinical isolates (one each of *A. fumigatus*, *Mucor* sp., and *S. prolificans* and two of *Fusarium* spp.) demonstrated SAAS MICs of POS and ITZ that were ≤0.25 μg/ml and correlated with low MICs as measured by the NCCLS method (Tables 1 and 2).

Our study describes the first evaluation of a screening antifungal susceptibility test for filamentous fungi. In preliminary studies, the semisolid HI medium supported the growth of a variety of filamentous fungi, and growth inhibition occurred when antifungal agents were added (9). We therefore studied four antifungal agents (AMB, ABEL, ITZ, and POS) against 54 clinical isolates and compared our results with those obtained by the NCCLS broth microdilution reference method which was proposed at the time. Our results show that of the more than 50 clinical mold isolates tested by the SAAS and NCCLS methods, most demonstrated susceptibility to all four antifungal agents. Testing with two different media, RPMI 1640 and AM3, in the NCCLS protocol did not produce significantly different results for AMB. For these isolates, results in AM3 medium were equal to or 1 dilution lower than those in RPMI 1640. Utilizing an endpoint of ≥50% growth reduction, POS and ITZ exhibited excellent activity against most isolates, including the non-*Aspergillus* species. Concordance between the two methods was high for all drugs tested.

The SAAS screening test differs from the NCCLS reference standard antifungal susceptibility test in two major ways: it uses a chemically undefined medium and an inoculum of mostly conidia that is not standardized to conidial size of the organism. As such, the SAAS test is not meant to replace the NCCLS test. In this era of newer and different antifungal agents, it was designed to preliminarily screen for antifungal drug resistance of invasive clinical fungal pathogens while identification and susceptibility testing are being carried out at reference laboratories.

HI broth is a chemically undefined medium that was chosen for this screening test to reproduce to some degree the expected growth conditions in an infected state where concentrations of oxygen, glucose, and minerals are likely reduced. Others have shown that the RPMI 1640 medium used in the NCCLS protocol for the reference standard test does not support the growth of some filamentous fungi as well as chemically undefined media do (D. T. A. te Dorsthorst, M. Janssen, A. J. M. M. Rijs, and P. Verweij, Abstr. 42nd Intersci. Conf. Antimicrob. Agents Chemother., abstr. M-1484, 2002). Whether an undefined medium that supports the growth of filamentous fungi in vitro is superior to a chemically defined one such as RPMI 1640 for determining antifungal susceptibility is not known. The HI broth with agar, formulated to produce a semisolid agar medium used in the SAAS test, appears to support hyphal growth of the filamentous fungi within the agar. Although speculative, since hyphae are observed in growth patterns of the organisms in vivo, the hyphal growth pattern of filamentous fungi in semisolid agar might reflect the organism's in vivo metabolic state. This observation notwithstanding, clinical correlation with drug inhibition of hyphal growth in agar observed by the SAAS test is needed.

Investigators who developed the NCCLS M38-P reference standard for susceptibility testing of filamentous fungi found that inoculum density could be reliably reproduced with spectrophotometric adjustment of conidial suspensions and that conidial size affected the MICs of certain drugs and for certain

TABLE 3. Concordance between results of NCCLS M38-P and SAAS tests (within 3 drug dilutions) for each drug and medium tested

Drug	Medium	% Concordance
AMB	AM3	94
AMB	RPMI 1640	94
ABEL	AM3	85
ABEL	RPMI 1640	83
ITZ	RPMI 1640	94
POS	RPMI 1640	94

AMB, amphotericin B; ABEL, amphotericin B lipid complex; ITZ, itraconazole; POS, posaconazole.

organisms (3, 4). This observation led to the recommendation for spectrophotometric adjustment according to conidial size of the organism. Unfortunately, most clinical microbiology laboratories do not have the capacity to perform this test in a timely manner. Our aim was to develop a simple test that could be performed in the clinical microbiology laboratory once the organism was isolated to screen for resistant organisms while awaiting species identification. To reduce the time required for antifungal susceptibility screening of filamentous fungi, we used inoculum suspensions of mostly conidia that can be readily prepared from the original pure plate.

A limitation of our study was the lack of documentation at the time of the type of inocula prepared (conidial versus mixed hyphae and conidia) for the SAAS method. Despite this, MICs achieved by the SAAS test were comparable to those obtained by the NCCLS test. Since the SAAS test is meant to be used as a screening test, rigorous standardization of the inoculum may be less critical. Moreover, replicate testing of the same organisms with new inoculum preparations performed in the same manner has not shown variation in results (data not shown). Ultimately, clinical correlation studies will be needed to verify the validity of filamentous fungi inocula prepared in this manner.

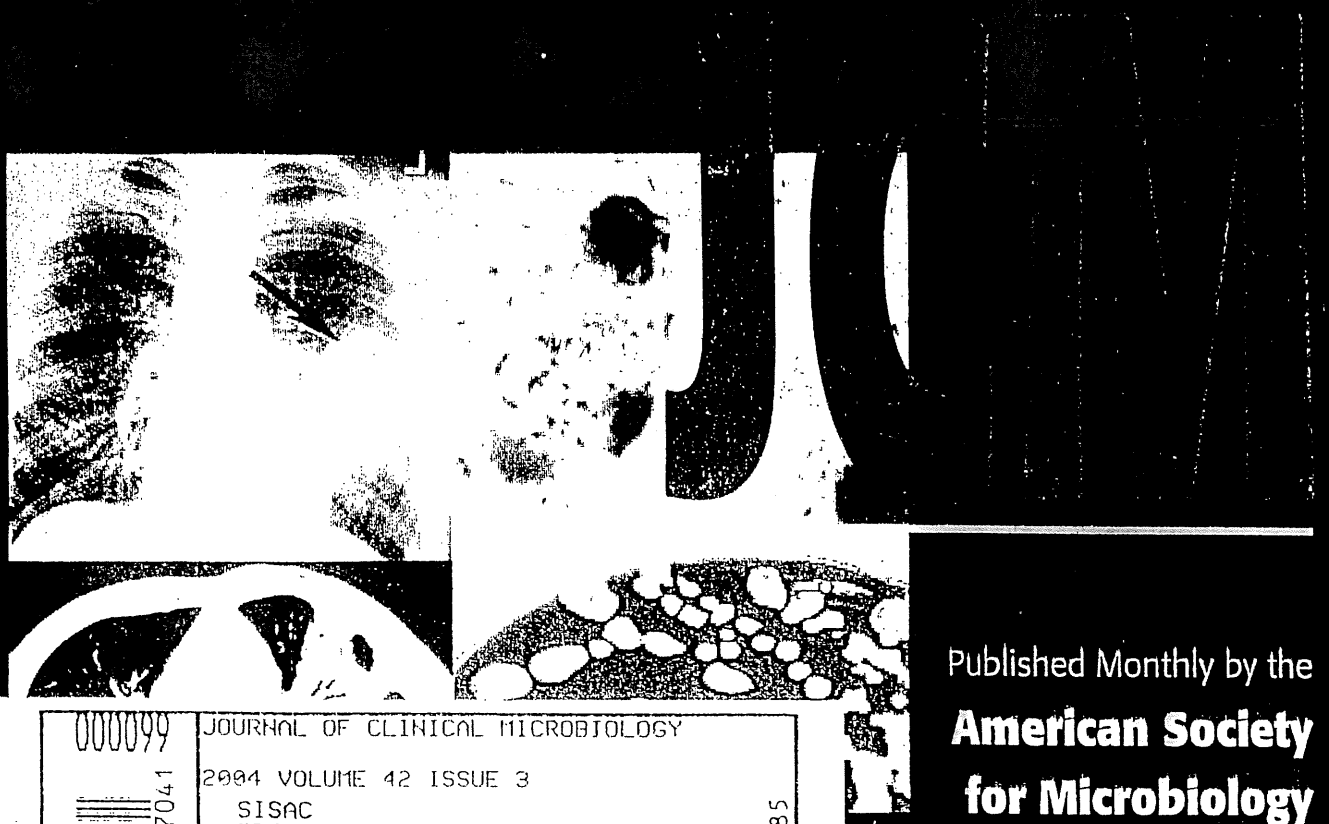
The SAAS test was developed as a screening antifungal susceptibility test and as such is not meant to determine MICs. Yet, to test its validity, MIC comparisons were made with the NCCLS test results. For the four antifungal agents tested, the concordance of results (within 1 dilution) between the two methods was high. These results suggest that the SAAS test accurately compares to the NCCLS method for prediction of susceptibility or resistance of the organism to the drug tested. In the future, SAAS testing of one drug concentration at the proposed cutoff for resistance would further simplify this screening test and could be considered once MIC endpoints for all drugs are determined for the filamentous fungi. Finally, we have previously demonstrated the utility of the SAAS test in real-time antifungal susceptibility screening for invasive candidiasis and its role in the clinical management of immunocompromised patients (5). Although this study was not designed to assess the correlation of MIC results and clinical outcome, the SAAS test predicted pan-resistance in the one patient with *S. prolificans* infection even before the identification of the organism was known. Future studies of real-time filamentous fungus susceptibility testing seem warranted.

In conclusion, we compared MIC results of the NCCLS M38-P broth microdilution method and the SAAS screening test for four antifungal agents tested against 54 clinical isolates of filamentous fungi. The SAAS test supported the growth of all filamentous fungi tested. We found excellent concordance of results for all four drugs tested. Most of the filamentous fungi were susceptible to both the azole and AMB preparations. Future studies comparing interlaboratory testing of a wider variety of filamentous fungi utilizing the SAAS and the recently approved NCCLS M38-A reference standard methods are warranted.

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