Comparative Evaluation of Etest and Sensititre YeastOne Panels against the Clinical and Laboratory Standards Institute M27-A2 Reference Broth Microdilution Method for Testing *Candida* Susceptibility to Seven Antifungal Agents[▽]

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Received 5 September 2006/Returned for modification 17 October 2006/Accepted 21 December 2006

To assess their utility for antifungal susceptibility testing in our clinical laboratory, the Etest and Sensititre methods were compared with the Clinical and Laboratory Standards Institute (CLSI) M27-A2 reference broth microdilution method. Fluconazole (FL), itraconazole (I), voriconazole (V), posaconazole (P), flucytosine (FC), caspofungin (C), and amphotericin B (A) were tested with 212 *Candida* isolates. Reference MICs were determined after 48 h of incubation, and Etest and Sensititre MICs were determined after 24 h and 48 h of incubation. Overall, excellent essential agreement (EA) between the reference and test methods was observed for Etest (95%) and Sensititre (91%). Etest showed an $\geq 92\%$ EA for MICs for all drugs tested; Sensititre showed a $\geq 92\%$ EA for MICs for I, FC, A, and C but 82% for FL and 85% for V. The overall categorical agreement (CA) was 90% for Etest and 88% for Sensititre; minor errors accounted for the majority of all categorical errors for both systems. Categorical agreement was lowest for *Candida glabrata* and *Candida tropicalis* with both test systems. Etest and Sensititre provided better CA at 24 h compared to 48 h for *C. glabrata*; however, CA for *C. glabrata* was $<80\%$ for FL with both test systems despite MIC determination at 24 h. Agreement between technologists for both methods was $\geq 98\%$ for each agent against all organisms tested. Overall, Etest and Sensititre methods compared favorably with the CLSI reference method for determining the susceptibility of *Candida*. However, further evaluation of their performance for determining the MICs of azoles, particularly for *C. glabrata*, is warranted.

Fungi have become pathogens of increasing concern over the past several decades, and *Candida* is now a common cause of nosocomial bloodstream infection in the United States (6). Accordingly, antifungal prophylaxis is routinely used in several populations considered at high risk for candidiasis, and breakthrough infections in patients on such prophylactic regimens are increasingly reported. The breakthrough organisms often have higher MICs of the drug to which they were exposed, and some may exhibit heteroresistance (14–16) and cross-resistance to one or more additional agents (1, 11, 33, 34). Although fluconazole and, more recently, voriconazole, have been widely used as prophylactic agents, the introduction of newer agents and several supporting recent studies will likely increase the spectrum of agents used for this purpose. Micafungin, a member of the echinocandin class of antifungal agents, has now been cleared by the U.S. Food and Drug Administration (FDA) for prophylactic use against *Candida* in hematopoietic stem cell transplant recipients (32). Posaconazole has also been approved by the FDA for prophylaxis in patients with graft-versus-host disease and in high-risk neutropenic patients after chemotherapy (A. J. Ullmann, J. H. Lipton,

D. H. Vesole, P. H. Chandrasekar, A. Langston, S. Tarantolo, H. Greinix, W. Azevedo, V. Reddy, C. Hardalo, H. Patino, and S. Durrant, Abstr. 45th Intersci. Conf. Antimicrob. Chemother., abstr. M716, 2005). The increasing number of antifungal agents now on the market, the widespread use of these agents in prophylactic regimens, and the frequent isolation of *Candida* species with unpredictable patterns of susceptibility to these agents has led clinicians to seek guidance from the clinical laboratory when selecting antifungal therapy for these complex, infected patients (26).

The Clinical and Laboratory Standards Institute (CLSI; formerly the National Committee for Clinical Laboratory Standards [NCCLS]) has developed and published an approved reference method for broth microdilution testing (CLSI document M27-A2) of *Candida* species. This method was developed through a consensus process to facilitate agreement among laboratories in determining the susceptibility of *Candida* species to several antifungal agents (20). This test method was published as a reference standard for use in clinical laboratories in 1997, updated as M27-A2 in 2002, and remains the standard method of susceptibility testing in many clinical laboratories. Unfortunately, this reference method is labor-intensive. Commercial systems are now available to clinical laboratories for determining MICs of various agents against *Candida* species. In November 2001, the Sensititre YeastOne test panel (Trek Diagnostic Systems, Inc., Westlake, OH) was cleared for commercial sale by the FDA as a new and less labor-intensive

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[▽] Published ahead of print on 3 January 2007.

method for determining MICs of fluconazole, itraconazole, and flucytosine for yeasts (8). Etest (AB BIODISK, Solna, Sweden), an agar-based predefined concentration gradient method for determining the MICs of various agents, is also available for *Candida* and could reduce further the effort required for routine susceptibility testing (17, 30).

The most important feature of a laboratory test for susceptibility testing is its ability to produce accurate results consistently within an appropriately rapid turnaround time in order to influence clinical decisions. Initial evaluations of these parameters are often performed in specialty or research laboratories by personnel with expertise in the test application area. Even FDA clearance of a commercial product does not predict how well that product will perform in an individual laboratory under actual testing conditions and with the clinical specimen mix encountered in particular hospital populations. Therefore, before one of the new antifungal susceptibility test devices can be used for clinical testing in place of the CLSI M27-A2 reference standard, a detailed comparative evaluation of its performance should be investigated by the offering laboratory. This local validation and verification of new test devices is a necessary part of the quality assurance program for all clinical laboratories in order to meet accreditation standards as required by the Centers for Medicare and Medicaid Services (4).

This single-center study was designed to compare the Etest and Sensititre antifungal susceptibility test systems with the CLSI M27-A2 reference method for seven antifungal agents in a hospital clinical laboratory as required by regulatory agencies. The present study is one of the first in a clinical laboratory setting to document the performance of these new test systems for seven of the most frequently used antifungal agents.

MATERIALS AND METHODS

Isolates. Duke University Health System Institutional Review Board approval was obtained for the present study. All patients with *Candida* bloodstream infection at Duke University Medical Center in 2000 and 2001 were identified via a review of the clinical microbiology laboratory database. A new episode of infection was defined as the recovery of the same *Candida* species more than 14 days after the last positive blood culture or if a new species was found ≥ 7 days since the last positive blood culture for *Candida*. A total of 212 initial isolates recovered from unique, consecutive episodes of bloodstream infection were retrieved from frozen (-70°C) storage for testing. Prior to testing, each isolate was subcultured at least twice on potato-dextrose agar to ensure purity and viability. All isolates were subcultured again 24 h prior to testing.

Antifungal agents. Caspofungin, voriconazole, and posaconazole research powders were provided by Merck Research Laboratories (Rahway, NJ), Pfizer Laboratories (Sandwich, Kent, United Kingdom), and Schering-Plough Research Institute (Kenilworth, NJ), respectively. Fluconazole, flucytosine, itraconazole, amphotericin B, voriconazole, posaconazole, and caspofungin Etest strips were provided by AB BIODISK. Sensititre YeastOne panels containing fluconazole, voriconazole, itraconazole, amphotericin B, flucytosine, and caspofungin were provided by Trek Diagnostic Systems.

Definitions. Standard antimicrobial susceptibility testing definitions were used (7, 18). For MICs measured with a continuous concentration scale (i.e., Etest) that yielded results falling in between conventional serial twofold dilutions, the next higher dilution was assigned. The results were considered to be in essential agreement when the test result was within two dilutions of the reference value. The percent essential agreement was calculated by using the number of test results in essential agreement as the numerator and the total number of organisms tested as the denominator. The results were considered to be in categorical agreement when the test and reference MICs fell within the same interpretive category (i.e., susceptible, susceptible dose dependent, intermediate, or resistant, depending on the agent tested). Established CLSI interpretive breakpoints were used to tabulate categorical agreement and very major, major, and minor errors for fluconazole, itraconazole, and flucytosine; proposed interpretive breakpoints

were used for voriconazole (20, 23). The percent categorical agreement was calculated by dividing the number of tests with no category discrepancy by the number of organisms tested. "Very major errors" occurred when the reference method categorized the organism as resistant, but the test method categorized the organism as susceptible (falsely susceptible). The percent very major errors were calculated by using the number of resistant isolates as the denominator. "Major errors" occurred when the reference method categorized the isolate as susceptible, but the test method categorized it as resistant (falsely resistant) and were calculated by using the number of susceptible isolates as the denominator. "Minor errors" occurred when the reference method categorized an organism as susceptible or resistant and the test method categorized it as susceptible dose dependent (or intermediate) or the reference categorized it as susceptible dose dependent (or intermediate) and the test method categorized it as susceptible or resistant. The percent minor errors were calculated by using the total number of organisms tested as the denominator.

Testing. Each *Candida* isolate was tested with the three methods in parallel as outlined below by two fully certified (American Society of Clinical Pathologists) medical technologists (B. D. Alexander and T. C. Byrne) with training in antifungal susceptibility testing. All seven antifungal agents were tested with each isolate by all test systems with the exception of posaconazole, which was not included on the Sensititre panels. Technologists interpreted results from each test system independent of the other test system results and independent of each other. The results were recorded on paper onto a preset grid, and a database expert (K. L. Smith) subsequently entered these results into an electronic database. Testing was repeated for tests with major and very major errors (for drugs with categorical interpretive criteria available) and for MIC results that did not agree with the reference standard within two dilutions. Discrepancies were resolved through repeat testing of both systems (the test method and reference method) in triplicate.

Inoculum suspension. Yeast inoculum suspensions were prepared as described in the CLSI M27-A2 document using sterile 0.85% saline (20). The cell density was adjusted with a spectrophotometer by adding sufficient saline to match the transmittance produced by a 0.5 McFarland density standard at a 530-nm wavelength, resulting in a concentration of 1×10^6 to 5×10^6 cells per ml. This suspension was used to directly inoculate agar plates for Etest and was diluted as directed by CLSI M27-A2 for the broth microdilution procedure. For the Sensititre system, a suspension of yeast in demineralized water was adjusted to match the turbidity of a 0.5 McFarland standard by using a Sensititre nephelometer; 20 μl of this suspension was transferred into an 11-ml tube of Sensititre YeastOne inoculum broth, resulting in a concentration of 1.5×10^3 to 8×10^3 cells per ml. This broth suspension was then used to inoculate the wells of the Sensititre plates.

CLSI M27-A2 broth microdilution method. The CLSI M27-A2 broth microdilution method was performed in accordance with the published guidelines that specify RPMI 1640 medium (Remel, Lenexa, KS) buffered to pH 7.0 with MOPS (morpholinepropanesulfonic acid) (20). Microdilution trays were incubated at 35°C and read visually after 48 h of incubation. For amphotericin B, the MIC endpoint was defined as the lowest concentration with complete (100%) growth inhibition. For the azoles, flucytosine, and caspofungin, an 80% inhibition in growth compared to that of the drug-free growth control was used as the MIC endpoint.

Etest predefined concentration gradient method. The Etest method was performed according to the manufacturer's instructions. Preformed and predefined gradients of the different agents immobilized on individual plastic test strips covered a continuous concentration range across 15 dilutions. A cotton-tipped, sterile swab was used to inoculate the organism from the yeast suspension onto a 150-mm agar plate containing RPMI 1640 medium supplemented with 2% glucose and buffered with MOPS to pH 7.0. After allowing the excess moisture to be fully absorbed into the agar, Etest strips were applied to the inoculated surface. The plates were incubated at 35°C and read at 24 and 48 h. The MIC was read as the lowest concentration at which the border of the elliptical zone of growth inhibition intersected the scale on the test strip. For the azoles and caspofungin an 80% inhibition in growth was used as the MIC cutoff (microcolonies were ignored), and for flucytosine and amphotericin B the MIC endpoint was defined as the lowest concentration with nearly complete (90%) and complete (100%) growth inhibition, respectively.

Sensititre YeastOne colorimetric antifungal susceptibility test method. The Sensititre antifungal susceptibility test method was performed according to the manufacturer's instructions. Plates containing serial twofold dilutions of the antifungal agents across 12 dilutions were inoculated using a suspension of the organism prepared as described above, incubated at 35°C , and read at 24 and 48 h. The MIC was recorded as the lowest concentration of antifungal agent preventing the development of a red color (the first blue well).

TABLE 2. Distribution of 212 organisms tested according to species and susceptibility profile^a

<i>Candida</i> species ^b	No. (%) of isolates			
	Total	S	S-DD (I) ^c	R
<i>C. albicans</i>	94 (44)			
Fluconazole		89 (95)	3 (3)	2 (2)
Itraconazole		88 (94)	2 (2)	4 (4)
Voriconazole		92 (98)	0	2 (2)
Flucytosine		91 (97)	0	3 (3)
<i>C. glabrata</i>	38 (18)			
Fluconazole		19 (50)	16 (42)	3 (8)
Itraconazole		1 (3)	12 (31)	25 (66)
Voriconazole		35 (92)	1 (3)	2 (5)
Flucytosine		36 (95)	0	2 (5)
<i>C. tropicalis</i>	34 (16)			
Fluconazole		32 (94)	0	2 (6)
Itraconazole		25 (73)	6 (18)	3 (9)
Voriconazole		31 (91)	0	3 (9)
Flucytosine		33 (97)	0	1 (3)
<i>C. parapsilosis</i>	31 (15)			
Fluconazole		30 (97)	0	1 (3)
Itraconazole		26 (84)	5 (16)	0
Voriconazole		30 (97)	1 (3)	0
Flucytosine		31 (100)	0	0
<i>C. krusei</i>	5 (2)			
Fluconazole		0	3 (60)	2 (40)
Itraconazole		0	5 (100)	0
Voriconazole		5 (100)	0	0
Flucytosine		0	4 (80)	1 (20)
<i>C. lusitanae</i>	8 (4)			
Fluconazole		7 (88)	1 (12)	0
Itraconazole		8 (100)	0	0
Voriconazole		8 (100)	0	0
Flucytosine		8 (100)	0	0
<i>C. guilliermondii</i>	2 (1)			
Fluconazole		2 (100)	0	0
Itraconazole		0	2 (100)	0
Voriconazole		2 (100)	0	0
Flucytosine		2 (100)	0	0

^a S, susceptible; S-DD, susceptible dose dependent; I, intermediate; R, resistant. Values were as determined by the CLSI M27-A2 broth microdilution reference method.

^b Proposed breakpoints were used to determine interpretive category for voriconazole (23).

^c I, intermediate interpretive category for flucytosine only.

Candida species with all antifungal agents are summarized in Table 5. Low percent categorical agreements for *C. krusei*, *C. lusitanae*, and *C. guilliermondii* were possibly exaggerated by the relatively small number of these *Candida* species tested. Otherwise, *C. glabrata* and *C. tropicalis* showed the lowest rates of categorical agreement in both test systems; categorical agreement was lowest for *C. glabrata* to fluconazole and *C. glabrata* and *C. tropicalis* to itraconazole for both test systems and *C. parapsilosis* to itraconazole for Sensititre. The percent essential and categorical agreements at 24 h were compared to percent essential and categorical agreements at 48 h for Etest and Sensititre for the azole antifungals. When organisms with insufficient growth at 24 h were excluded from the analysis, the overall categorical agreement was not significantly different at the 24- and 48-h readings for Etest ($P = 0.141$); for Sensititre, significantly better categorical agreement was obtained with 24-h readings ($P = 0.005$) (Table 6). Similar results were obtained when the 24-h versus the 48-h readings for fluconazole and itraconazole were assessed independently (data not shown).

For *C. glabrata*, there was no significant difference in the percent essential agreement between the 24- and 48-h readings with Etest; however, the categorical agreement was improved at 24 h ($P = 0.024$). For Sensititre, significantly better essential ($P = 0.0001$) and categorical ($P = 0.005$) agreements were achieved with 24 h compared to the 48-h results. For *C. tropicalis*, reading at 48 h provided significantly better essential agreement than reading at 24 h ($P = 0.0001$) for Etest, although this provided no improvement in categorical agreement. For the Sensititre system, no significant difference in essential or categorical agreement was observed between the 24- and 48-h readings.

Minor errors accounted for the majority of all categorical errors for both systems. For *C. glabrata* and *C. parapsilosis*, 100% of errors for itraconazole were minor with both test systems; for *C. tropicalis*, 92 and 91% of errors for itraconazole were minor with Etest and Sensititre, respectively. For *C. glabrata*, 88 and 96% of the errors for fluconazole were minor for Etest and Sensititre, respectively. Only two very major errors were documented. Both very major errors occurred with the Etest system for different isolates of *C. tropicalis*: one with itraconazole and one with voriconazole.

Agreement between technologists for both the Etest and the Sensititre methods was 98% or greater for each antifungal agent against all organisms tested. Of the 1,484 total tests

TABLE 3. Summary of MIC₉₀ values determined by CLSI M27-A2 broth microdilution, Etest, and Sensititre test methods at 48 h

Drug	MIC (μg/ml)					
	CLSI microdilution		Etest		Sensititre	
	MIC ₉₀	Range	MIC ₉₀	Range	MIC ₉₀	Range
Amphotericin B	1	0.125–2	1	0.125–4	2	0.25–4
Caspofungin	1	<0.03–2	0.5	0.03–8	0.5	0.03–4
Fluconazole	16	<0.03–>64	32	0.06–256	64	<0.125–>256
Itraconazole	2	<0.03–16	4	0.016–32	2	0.008–>16
Voriconazole	0.5	<0.03–>64	1	0.008–32	1	<0.008–16
Posaconazole	1	<0.03–16	2	0.016–32	NA ^a	NA
Flucytosine	1	<0.03–>64	4	0.008–32	0.5	<0.03–16

^a NA, not applicable.

TABLE 4. Agreement by drug: Etest and Sensititre are compared with the CLSI M27-A2 broth microdilution reference method^a

Drug tested	Etest					Sensititre				
	No. (%) of category errors				No. (%) EA	No. (%) of category errors				No. (%) EA
	Minor	Major	Very major	No error CA		Minor	Major	Very major	No error CA	
Fluconazole	23 (11)	2 (1)	0	187 (88)	195 (92)	33 (16)	1 (1)	0	176 (83)	174 (82)
Itraconazole	41 (19)	0	1 (3)	170 (80)	202 (95)	55 (26)	2 (1)	0	155 (73)	204 (96)
Voriconazole	9 (4)	0	1 (14)	202 (95)	195 (92)	4 (2)	2 (1)	0	206 (97)	180 (85)
Flucytosine	5 (2)	2 (1)	0	205 (97)	203 (96)	2 (1)	0	0	210 (99)	194 (92)
Posaconazole	NA	NA	NA	NA	210 (99)	NA	NA	NA	NA	NA
Amphotericin B	NA	NA	NA	NA	211 (99)	NA	NA	NA	NA	212 (100)
Caspofungin	NA	NA	NA	NA	195 (92)	NA	NA	NA	NA	194 (92)
Overall (%)	9	0.5	4	90	95	11	0.7	0	88	91

^a No. (%) EA, discrepancies between MIC endpoint determinations of no more than two doubling dilutions were used to calculate the percent essential agreement (% EA). CA, categorical agreement. NA, not applicable. Major, calculations include only isolate susceptible by CLSI M27-A2 broth microdilution reference method. Very major, calculations include only isolates resistant by CLSI M27-A2 broth microdilution reference method.

interpreted by using the Etest, eight disagreements of more than two doubling dilutions were documented, resulting in 99.46% essential agreement overall (95% CI = 98.94 to 99.77%). For Sensititre, of 1,272 tests interpreted, five disagreements were recorded, resulting in 99.61% essential agreement overall (95% CI = 99.09 to 99.87%).

DISCUSSION

The goal of the present study was to validate the performance in our laboratory of two new commercially available systems for antifungal susceptibility testing before implementing the test systems for clinical use. In our hands, the overall percent essential agreement for the Etest and Sensititre systems was >90%. When discrepancies compared to the CLSI method were found, Etest tended to yield higher MICs for all drugs except caspofungin, whereas Sensititre tended to yield higher MICs for all drugs tested except for caspofungin and flucytosine. The essential agreement was >90% for all drugs tested, except for fluconazole and voriconazole with the Sen-

sititre system. Despite only 85% overall essential agreement for voriconazole with the Sensititre system, the categorical agreement was still 97%. On the other hand, the lack of essential agreement for fluconazole with the Sensititre system did lead to lower categorical agreement (83%) for this drug.

In general, the number of categorical errors was low for both test systems for the four agents with CLSI MIC breakpoints. When categorical errors did occur, minor errors accounted for the majority. We observed two very major (falsely susceptible) errors for the Etest. The purpose for calculating very major errors is to determine whether a test system can detect resistance since failure to do so could place a patient in jeopardy. The criteria for categorical errors used by the FDA in considering a susceptibility test system for clearance specify $\leq 1.5\%$ very major errors and $\leq 3\%$ major errors (10). For a clinical laboratory and test system to meet this stringent requirement for very major errors, at least 65 isolates resistant to each drug under study would need to be tested to allow one very major error per drug tested and still have an acceptable error rate of

TABLE 5. Agreement by *Candida* species: Etest and Sensititre compared with CLSI M27-A2 broth microdilution reference method^a

Test system	<i>Candida</i> species	No. of isolates	% Categorical agreement					% Essential agreement							
			5FC	FLC	ITC	VRC	Overall	5FC	FLC	ITC	VRC	PSC	AMB	CSP	Overall
Etest	<i>C. albicans</i>	94	97	98	94	100	97	93	98	97	93	100	99	89	96
	<i>C. glabrata</i>	38	100	55	74	76	76	100	82	89	89	95	100	90	92
	<i>C. tropicalis</i>	34	100	97	62	97	89	94	100	94	85	100	100	88	94
	<i>C. parapsilosis</i>	31	100	97	81	100	94	100	87	100	97	100	100	100	98
	<i>C. krusei</i>	5	20	40	0	100	40	100	40	80	100	100	100	100	89
	<i>C. lusitaniae</i>	8	100	88	88	100	94	100	100	100	100	100	100	100	100
	<i>C. guillermundii</i>	2	100	100	50	100	88	100	100	100	100	100	100	100	100
	Overall	212	97	88	80	95	90	96	92	95	92	99	99	92	95
Sensititre	<i>C. albicans</i>	94	99	97	100	100	99	96	96	100	82	NA	100	90	94
	<i>C. glabrata</i>	38	100	34	68	87	72	97	66	89	89	NA	100	97	90
	<i>C. tropicalis</i>	34	100	94	32	97	81	79	85	88	76	NA	100	79	85
	<i>C. parapsilosis</i>	31	100	90	48	100	85	87	48	100	90	NA	100	97	87
	<i>C. krusei</i>	5	80	40	80	100	75	100	100	100	100	NA	100	100	100
	<i>C. lusitaniae</i>	8	100	100	50	100	88	100	100	100	100	NA	100	100	100
	<i>C. guillermundii</i>	2	100	100	50	100	88	0	100	100	100	NA	100	100	83
	Overall	212	99	83	73	97	88	92	82	96	85	NA	100	92	91

^a FLC, fluconazole; PSC, posaconazole; ITC, itraconazole; VRC, voriconazole; 5FC, 5 flucytosine; AMB, amphotericin B; CSP, caspofungin. NA, not applicable.

TABLE 6. Etest and Sensititre compared with the CLSI M27-A2 reference method: 24-h versus 48-h agreement results for azole antifungals^a

Organism	Test system	% CA ^b				% EA			
		No. TYTR	24 h	48 h	P	No. TYTR	24 h	48 h	P
All <i>Candida</i> species	Etest	17	90	88	0.141	23	93	94	0.162
	Sensititre	61	87	85	0.005	61	89	87	0.171
<i>Candida glabrata</i>	Etest	5	81	68	0.024	6	93	88	0.189
	Sensititre	10	77	62	0.005	10	97	80	0.0001
<i>Candida tropicalis</i>	Etest	0	85	86	1.000	0	85	95	0.0001
	Sensititre	3	78	76	0.754	3	91	83	0.077

^a The azole antifungals tested with Etest included fluconazole, itraconazole, voriconazole, and posaconazole; azole antifungals tested with Sensititre included fluconazole, itraconazole, and voriconazole. TYTR, too young to read (number of organisms with insufficient growth to read MIC endpoint at 24 h). For the *P* values, the percent categorical agreement (CA) and percent essential agreement (EA) at 24 h and 48 h were compared using McNemar's chi-square test and excluded isolates TYTR at 24 h.

^b The % CA does not include posaconazole for the Etest since no interpretive breakpoints are currently approved.

<1.5% (7, 18). Our cohort included 212 isolates with 56 total resistant results (the numbers of isolates resistant to fluconazole, itraconazole, voriconazole, and flucytosine were 10, 32, 7, and 7, respectively). When 65 isolates resistant to each drug are not available for testing as in our study, experts have suggested that a less stringent criterion be applied (e.g., the total of the very major and major errors should be <5%) (12). Regardless of the exact criterion used, it is important to examine a collection of challenge strains that contains enough resistant isolates to verify the ability of the new test to detect resistance. We tested a relatively large cohort of isolates with 56 instances of resistance to one or more drugs; the Etest system failed to detect resistance only twice. The Sensititre system did not fail to detect resistance.

Importantly, both very major errors for the Etest system were found in testing *C. tropicalis* against azole antifungals. One potential explanation is related to known limitations of the reference method. Trailing endpoints, a well-described phenomenon with azole antifungals and the CLSI M27-A2 microdilution method, can lead to overinterpretation of the MICs, particularly for *C. tropicalis*. In six of the seven discrepancies between Etest and CLSI M27-A2 azole results for *C. tropicalis*, the MIC was lower by Etest. For Sensititre, also a microdilution method, only 1 of the 13 discordant MIC azole results for *C. tropicalis* was lower than the CLSI M27-A2 result. Thus, it is plausible that improved endpoint discrimination by Etest accounts for its discrepancies with the CLSI reference method.

Of the drugs tested, itraconazole and fluconazole were associated with the lowest rates of overall categorical agreement in both test systems. Furthermore, *C. glabrata* had high rates of nonconcordance for fluconazole and itraconazole compared to other species, and *C. tropicalis* had high rates of nonconcordance with itraconazole. These findings are important given that one of the most clinically pertinent and frequent reasons to perform antifungal susceptibility testing is the need to determine the susceptibility of non-*albicans* *Candida* species to the azoles. For all discrepant results for *C. glabrata* and an azole, the MIC was higher by both test systems compared to the reference MIC. This raises the possibility that *C. glabrata* may be associated with azole resistance detectable by the test systems but undetectable by the reference method. Heteroresistance, or selection of a resistant subpopulation of cells from

a population of predominantly susceptible cells, could account for such a phenomenon. Heteroresistance has been described in both *Cryptococcus neoformans* and *C. albicans* (15, 16, 29). The molecular mechanism associated with heteroresistance in *C. albicans*, i.e., the upregulation of CDR genes responsible for encoding cell wall pumps that infer cross-resistance among azoles, has also been associated with the development of fluconazole resistance in *C. glabrata* (3, 27, 28). Further, detection of the heterogenous phenotype in strains of *C. albicans* was possible by using Etest; heterogenous isolates displayed increased satellite growth in the inner zone of growth inhibition, while homogeneous isolates displayed clearly demarcated zones of inhibition (15). It is therefore feasible that the test methods may be better able to detect heteroresistance than the reference method.

Other investigators have also found lower rates of agreement of *C. glabrata* and *C. tropicalis* for azoles when comparing the Etest and Sensititre systems to the CLSI M27-A2 method. Moreover, incubation times have been shown to significantly influence MIC results. Based on the current literature, however, it is not clear whether reading endpoints at 24 h provides better agreement than reading at 48 h for the different species of *Candida*. For instance, in a study of fluconazole that compared Etest with CLSI M27-A2, there was better essential agreement at 24 h than at 48 h for *C. tropicalis* and *C. albicans*, but lower agreement at both times for *C. glabrata* (30). In a study of posaconazole, *C. glabrata* and *C. tropicalis* showed the lowest concordance rates between Etest and CLSI M27-A2 at both times. However, *C. glabrata* essential agreement was higher at 24 h (72%) than at 48 h (56%), whereas *C. tropicalis* was higher at 48 h (71%) than at 24 h (39%) (31). In studies comparing the Sensititre system with the CLSI M27-A2 method, agreement at 24 h compared to 48 h also seemed to vary for the different *Candida* species. In one study that defined essential agreement as within three dilutions of the reference standard, the best performance of the Sensititre system was with 24 h MICs (92 to 100% essential agreement) with the azoles and flucytosine for all of the species tested, with the exception of *C. albicans* (87 to 90%) (9). In another study, overall agreement between the Sensititre and CLSI M27-A2 methods was better at 24 h (95 to 99% agreement) than at 48 h (80 to 97% agreement) for all species and antifungal agents (posaconazole, ravuconazole, and voriconazole) because Sen-

sititre MICs were consistently more than two dilutions higher than the reference MICs, especially for isolates of *C. albicans*, *C. glabrata*, and *C. tropicalis* (8). Finally, in a study comparing the Sensititre to the CLSI M27-A2 method for voriconazole, agreement was <90% for *C. glabrata*, despite the 24-h readings (24). In the present study, we also compared essential and categorical agreement with 48-h CLSI reference results for the azole antifungals read at 24 h and at 48 h with Etest and Sensititre. We were unable to compare 24-h MICs obtained by the reference method with 24-h MICs obtained by the test systems since the 24-h CLSI MIC results were not recorded. For Etest, 24-h categorical agreement with the CLSI M27-A2 method was significantly better only for isolates of *C. glabrata*. For *C. tropicalis*, reading at 48 h actually provided significantly better essential agreement than reading at 24 h, although this provided no improvement in categorical agreement. For Sensititre, categorical agreement was significantly better at 24 h than at 48 h when data were analyzed for the entire cohort of isolates. When data for the various *Candida* species were analyzed separately, categorical agreement was better at 24 h for *C. glabrata* but not for *C. tropicalis*. Despite significant improvement in categorical agreement by reading MICs at 24 h for *C. glabrata* with both test systems, the categorical agreement for fluconazole to *C. glabrata* remained low (75% Etest, 62% Sensititre). In our opinion, further investigation with an even larger cohort of non-*albicans* species into the cause for these discrepancies is needed. In the meantime, for isolates with sufficient growth, reading MIC results at 24 h, particularly for *C. glabrata* to the azole antifungals, is suggested.

Despite widespread and growing use of echinocandins and the concomitant risk for development of drug resistance (5, 13), there is no current CLSI method for MIC determinations. One published study suggested that using RPMI medium, the lowest concentration of caspofungin resulting in a significant diminution of growth below control growth levels as the endpoint, and reading endpoints at 24 h provides the best interlaboratory agreement when testing caspofungin (21). Under these conditions, a high level of discrimination was achieved between strains known to have lowered caspofungin susceptibility (based on molecular evidence of mutations in the FKS₁ gene) and normally susceptible isolates. The range of caspofungin MICs in our cohort of pedigree isolates was <0.03 to 2 µg/ml, a finding consistent with ranges published by other investigators (22). Further, the MIC for the quality control isolates fell almost perfectly within expected limits, despite reading at 48 h. Our study suggests that the new test systems may provide results in concordance with those obtained with RPMI medium by the CLSI M27-A2 method in testing caspofungin.

In order to ensure general applicability of the study results, our selection of isolates for testing included a spectrum of *Candida* species typically isolated in a clinical microbiology laboratory and mirrored national and international epidemiological data (35). Moreover, a broad range of MICs was also exhibited by the isolates from blood cultures. For example, 50% of the *C. glabrata* blood isolates demonstrated an MIC of ≤8 µg/ml (susceptible), 42% demonstrated an MIC of 16 to 32 µg/ml (susceptible dose dependent), and 8% demonstrated an MIC of ≥64 µg/ml (resistant) to fluconazole. Nonetheless,

66% (25 of 38) of *C. glabrata* isolates tested were resistant to itraconazole, whereas only 8% (3 of 38) tested resistant to fluconazole based on current interpretive categorical breakpoints. Given drug utilization at our hospital, one might have expected fluconazole resistance to exceed itraconazole resistance for *C. glabrata* if this finding were attributable to treatment-induced resistance. This paradox has been observed in other epidemiologic studies as well (22). In a large study including 949 clinical isolates of *C. glabrata*, all of the 78 strains that were resistant to fluconazole were also resistant to itraconazole (25). Conversely, of the 567 isolates found to be susceptible to fluconazole, only 4% were susceptible to itraconazole (inhibited by itraconazole at an MIC of ≤0.12 µg/ml). Whether this finding reflects a unique mechanism for resistance to itraconazole that does not impart resistance to fluconazole in *C. glabrata*, inappropriate thresholds for categorical classifications of resistance for itraconazole, or inability of the reference method to detect resistance to fluconazole in *C. glabrata* is uncertain, but we suspect one of the latter.

In order to comply with the recommended guidelines for verification studies that use a reference method that is known to be an imperfect standard, discrepant test results were resolved by retesting in triplicate both the new test system and the reference method. Initial discrepancies that resolved with repeat testing included three major errors (all for flucytosine) with Etest and five major errors (two for fluconazole, two for itraconazole, and one for voriconazole) with Sensititre. All major error resolutions were due to changes in initial results with the Etest or Sensititre systems. Using initial test results for Etest, 0.9% major errors occurred compared to 0.5% major errors when repeat test results were used. Using initial test results for Sensititre, 1.4% major errors occurred compared to 0.7% major errors when repeat test results were used. Thus, using the initial test results, major errors were still within the acceptable overall categorical agreement for major errors (3%) with both test systems.

Seven very major error discrepancies (two with fluconazole, five with voriconazole) resolved with repeat testing for Etest and six very major error discrepancies (one with fluconazole, five with voriconazole) resolved with repeat testing for Sensititre. All very major error resolutions were due to changes in the initial CLSI reference method result. Interestingly, all but one of the very major discrepancies that resolved for both test systems were with the same isolates. This highlights known limitations of the current CLSI standard and supports the importance of repeating discrepancies in triplicate with both systems when performing verification studies for the clinical laboratory.

Clinical laboratories are playing an increasingly important role in antifungal drug selection by performance of susceptibility testing of *Candida* isolated from infected patients. It is essential that the antifungal drug testing systems used clinically provide accurate results when compared to the reference standard. The most important use of antifungal susceptibility testing in the clinical laboratory is to detect resistant isolates, and both Etest and Sensititre performed well in this regard. The methods also performed well for some of the newer antifungal agents including voriconazole, posaconazole, and caspofungin. Furthermore, interobserver variability between the two tech-

nologists for Etest and Sensititre methods was minimal, which shows that intralaboratory variability should be minimal with appropriate training. However, our study underscores the influence of incubation time on MIC results, especially with the azole agents. The Sensititre system provided better overall categorical agreement with the reference standard when there was sufficient growth for the results to be read at 24 h rather than at 48 h. For Etest, reading at 24 h also provided better categorical agreement for *C. glabrata*. However, despite improved agreement by reading at 24 h, categorical agreement for *C. glabrata* remained disappointingly low (<80% for fluconazole) with both test systems. Fortunately, the majority of errors encountered with the new test systems were minor, and both tended to overcall resistance for *C. glabrata* and thereby yield a conservative result for patient care. Nonetheless, because assessing fluconazole activity against *C. glabrata* is commonly needed clinically, further evaluation of both test systems (and the CLSI reference method and breakpoints) is warranted.

ACKNOWLEDGMENTS

This study was a component of NIH/NIAID K23-AI52222-01 (B.D.A.).

Testing supplies were provided by AB BIODISK and Trek Diagnostic Systems. Merck Research Laboratories, Pfizer Laboratories, and Schering-Plough Research Institute provided drugs for the investigation.

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