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Multisite Reproducibility of the Etest MIC Method for Antifungal Susceptibility Testing of Yeast Isolates

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A multicenter study was performed to establish the interlaboratory reproducibility of Etest, to provide an additional comparison of Etest MICs with reference broth macrodilution MICs, and to develop some tentative quality control (QC) guidelines for using Etest for antifungal susceptibility testing of *Candida* spp. Two QC strains, *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258, were tested by Etest against amphotericin B, fluconazole, flucytosine, itraconazole, and ketoconazole in each of four laboratories. The QC strains were tested 20 times each against the five antifungal agents by using a common lot of RPMI agar. A total of 80 MICs per drug per strain were generated during the study. Overall, 98 to 100% of the MICs fell within a 3 $\log_2$ dilution range for the respective yeast-antifungal agent combinations. The level of agreement of Etest MICs with broth macrodilution MICs was 86 to 100% with amphotericin B (*C. krusei* and *C. parapsilosis*), itraconazole (*C. krusei* and *C. parapsilosis*), flucytosine (*C. parapsilosis*), and fluconazole (*C. parapsilosis*). A lower level of agreement was observed with ketoconazole (*C. krusei* and *C. parapsilosis*). Although all participants reported identical Etest MICs, the MICs of flucytosine and fluconazole when tested against *C. krusei* fell well above the upper limits of the reference range for this strain. The tentative QC limits for the two QC strains and five antifungal agents when tested by the Etest methodology are the same as the QC limits when tested by the reference broth macrodilution method for amphotericin B and *C. krusei*, itraconazole and *C. krusei*, flucytosine and *C. parapsilosis*, fluconazole and *C. parapsilosis*, and itraconazole and *C. parapsilosis*. The Etest QC ranges are 1 dilution broader (4-dilution range) than the reference macrodilution method QC ranges for ketoconazole and *C. krusei*, amphotericin B and *C. parapsilosis*, and ketoconazole and *C. parapsilosis*.

Through a consensus process, the National Committee for Clinical Laboratory Standards (NCCLS) Subcommittee on Antifungal Susceptibility Testing has developed a standardized and reproducible method for broth dilution testing of yeasts (7). Although the reference M27-T macrodilution broth method has proven to be useful as a means of performing antifungal susceptibility testing, easier test procedures are desirable (5).

Several modifications of the reference broth macrodilution method are under investigation and offer promise as alternative approaches that may better serve practical clinical laboratory needs. These approaches include various microdilution methods (4, 9, 10, 12) and the Etest MIC method (2, 3, 12). Etest (AB Biodisk, Solna, Sweden) is based on the use of a continuous concentration gradient of an antimicrobial agent on a plastic strip transferred to an agar medium. Comparative evaluations of Etest versus broth macrodilution and broth microdilution susceptibility testing of various antifungal agents against clinical isolates of *Candida* species indicate that Etest is a promising method for performing antifungal susceptibility testing (2, 3, 6, 12, 13). The present multicenter study was performed to further establish the intra- and interlaboratory reproducibilities of Etest, to provide an additional comparison of Etest MICs with reference broth macrodilution MICs, and to develop some tentative quality control (QC) guidelines for using Etest for antifungal susceptibility testing of *Candida* spp.

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MATERIALS AND METHODS

Yeast isolates. The QC strains listed in the NCCLS M27-T document (7), *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019, were selected for testing.

Antifungal agents. Etest strips containing amphotericin B (concentration range, 0.002 to 32 μg/ml), flucytosine (0.002 to 32 μg/ml), fluconazole (0.016 to 256 μg/ml), itraconazole (0.002 to 32 μg/ml), and ketoconazole (0.002 to 32 μg/ml) were supplied by AB Biodisk.

Antifungal susceptibility test method. Each isolate was tested against the five antifungal agents by the Etest method as recommended by the manufacturer. Briefly, Etest was performed by inoculation of a 150-mm petri plate containing 60 ml of RPMI agar supplemented with 2% glucose and buffered to pH 7.0 with morpholinepropanesulfonic acid (Remel, Lenexa, Kans.). The inoculum was applied with a nontoxic swab by using a cell suspension in 0.85% NaCl; the cell suspension was adjusted to a 0.5 McFarland standard. The Etest strips were applied after the excess moisture had been absorbed into the agar. The plates were incubated at 35°C, and the results were read after 48 h.

Study design and analysis. Four laboratories participated in the study. Each laboratory received individual subcultures of the test isolates, Etest strips, and sufficient RPMI agar plates to perform the tests for the study. A single lot of RPMI agar plates was distributed to all participating laboratories. Each of the participating laboratories performed 20 replicate tests with each of the test strains against the five antifungal agents. Thus, a total of 80 MICs were available for each yeast and antifungal agent combination tested with Etest.

Interlaboratory agreement was determined by calculating the percentage of MICs within a 3-dilution range for each yeast-antifungal agent combination. In 7 of the 10 yeast-antifungal agent combinations, the 3-dilution range constituted the modal MIC ± 1 $\log_2$ dilution; however, in the remaining 3, which did not have a clear modal MIC, it was determined to be the 3-dilution range that encompassed the largest numbers of MICs reported (1). The reference macrodilution MIC ranges for each yeast-antifungal agent combination were defined in previous studies (8, 11). The agreement between the Etest method and the NCCLS M27-T macrodilution method was defined as the percentage of Etest

TABLE 1. Etest MICs of five antifungal agents for isolates of *C. krusei* and *C. parapsilosis* reported by four separate laboratories

Organism	Antifungal agent	No. of tests	MIC (μg/ml)		% MICs in each 3-dilution range
			Mode	Range encompassing ≥95% of values	
C. krusei ATCC 6258	Amphotericin B	80	1.0	0.5–2.0	100
	Flucytosine	80	≥32	≥32	100
	Fluconazole	80	≥256	≥256	100
	Itraconazole	80	0.5	0.25–1.0	100
	Ketoconazole	80	0.75	0.5–2.0	100
C. parapsilosis ATCC 22019	Amphotericin B	79[a]	0.75	0.5–2.0	99
	Flucytosine	80	0.5	0.25–1.0	99
	Fluconazole	80	2.0	2.0–8.0	98
	Itraconazole	80	0.125	0.06–0.25	100
	Ketoconazole	80	0.06	0.03–0.12	100

[a] One laboratory performed only 19 replicates.

MICs included in the macrodilution reference range for each yeast-antifungal agent combination.

RESULTS

Table 1 summarizes the in vitro susceptibilities of the two test isolates to amphotericin B, flucytosine, fluconazole, itraconazole, and ketoconazole as judged by the Etest method. A total of 799 Etest MICs were evaluated. Analysis of these MICs provides an estimate of intralaboratory reproducibility as well as interlaboratory agreement. A very tight distribution of MICs was observed with both isolates and all five antifungal agents. In the case of amphotericin B (*C. krusei* and *C. parapsilosis*), ketoconazole (*C. krusei* and *C. parapsilosis*), itraconazole (*C. krusei* and *C. parapsilosis*), and flucytosine (*C. parapsilosis*), 99 to 100% of the MICs fell within a 3-dilution range defined by the modal MIC $\pm$ 1 $\log_2$ dilution. The results for the other yeast-antifungal agent combinations were also highly reproducible: 98 to 100% of the MICs fell within a 3-dilution range for the respective combinations, but here the distribution of the MICs around the mode was generally skewed to one side. The 3-dilution range was therefore the one that encompassed the largest number of MICs. These results document the excellent intra- and interlaboratory reproducibilities of the Etest method.

The percentage of Etest MICs included in the macrodilution reference range for each species and antifungal agent is provided in Table 2. Overall, the levels of agreement by the macrodilution method were 86 to 100% with amphotericin B (*C. krusei* and *C. parapsilosis*), itraconazole (*C. krusei* and *C. parapsilosis*), flucytosine (*C. parapsilosis*), and fluconazole (*C. parapsilosis*). A lower level of agreement was observed with ketoconazole (*C. krusei* and *C. parapsilosis*); however, this was accounted for by a cluster of MICs that were either 1 dilution higher (*C. krusei*) or 1 dilution lower (*C. parapsilosis*) than the upper or lower limits of the respective reference ranges. Expansion of the ketoconazole reference ranges for *C. krusei* and *C. parapsilosis* by 1 dilution (0.12 to 1.0 and 0.03 to 0.25 μg/ml, respectively) would encompass 94 and 100% of the Etest MICs, respectively.

A complete lack of agreement of Etest MICs with reference broth macrodilution method MICs was noted when flucytosine and fluconazole were tested against *C. krusei* ATCC 6258. Although all participants reported identical Etest MICs, the MICs of these two antifungal agents fell well above the upper limits of the reference range for this strain. The reason for these differences is unclear; however, it may relate to differences in growth on glucose-supplemented agar medium versus growth in unsupplemented broth. It appears that there is a resistant subpopulation that is picked up by Etest but not by the reference broth macrodilution method. This particular

TABLE 2. Percent agreement of Etest method with reference broth macrodilution procedure

Organism	Antifungal agent	Reference procedure MIC range (μg/ml)[a]	% of MICs within the reference range	
			Macrodilution procedure[a]	Etest
C. krusei ATCC 6258	Amphotericin B	0.5–2.0	99	100
	Flucytosine	4.0–16	97	0
	Fluconazole	16–64	99	0
	Itraconazole	0.12–0.5	94	100
	Ketoconazole	0.12–0.5	100	49
C. parapsilosis ATCC 22019	Amphotericin B	0.25–1.0	99	86
	Flucytosine	0.12–0.5	99	98
	Fluconazole	2.0–8.0	99	98
	Itraconazole	0.06–0.25	99	100
	Ketoconazole	0.06–0.25	98	75

[a] Reported by Pfaller et al. (8) and Rex et al. (11).

TABLE 3. Tentative QC limits for five antifungal agents and two QC strains when tested by Etest

QC strain	Antifungal agent	QC MIC range (μg/ml [%[a]])	
		Reference macrodilution method[b]	Etest
C. krusei ATCC 6258	Amphotericin B	0.5–2.0	0.5–2.0 (100)
	Flucytosine	4.0–16	
	Fluconazole	16–64	
	Itraconazole	0.12–0.5	0.12–0.5 (100)
	Ketoconazole	0.12–0.5	0.12–1.0 (94)
C. parapsilosis ATCC 22019	Amphotericin B	0.25–1.0	0.25–2.0 (99)
	Flucytosine	0.12–0.5	0.12–0.5 (98)
	Fluconazole	2.0–8.0	2.0–8.0 (98)
	Itraconazole	0.06–0.25	0.06–0.25 (100)
	Ketoconazole	0.06–0.25	0.03–0.25 (100)

[a] Percentage of MICs in each range.
[b] Reported by Pfaller et al. (8) and Rex et al. (11).

strain of *C. krusei* is relatively resistant to both flucytosine and fluconazole when tested in broth, and the glucose-supplemented agar medium may further enhance its ability to grow in the presence of these drugs.

DISCUSSION

The results of the present study confirm our previous observations (3, 6, 12) and provide additional documentation of the level of agreement between the Etest method and the M27-T reference method for testing yeasts. Furthermore, the results provide additional evidence of the excellent intra- and interlaboratory reproducibilities of Etest for antifungal susceptibility (6).

Although *C. albicans* was not included in the present study, comparable performance may be expected with this species by the Etest methodology. Recently, we have demonstrated excellent intra- and interlaboratory reproducibilities with Etest for both reference (ATCC 90028) and clinical isolates of *C. albicans* (6). In the present study, we elected to test only the two NCCLS QC strains, *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258, in order to address the need of clinical laboratories for QC guidelines for the Etest methodology.

By using two well-characterized QC isolates in the present study, we have created an opportunity to establish some tentative QC limits for five antifungal agents when tested by the Etest methodology. These tentative QC limits are given in Table 3 and are the same as the reference broth macrodilution method QC limits for amphotericin B and *C. krusei*, itraconazole and *C. krusei*, flucytosine and *C. parapsilosis*, fluconazole and *C. parapsilosis*, and itraconazole and *C. parapsilosis*. The Etest QC ranges are 1 dilution broader (4-dilution range) than the reference broth macrodilution method QC ranges for ketoconazole and *C. krusei*, amphotericin B and *C. parapsilosis*, and ketoconazole and *C. parapsilosis*. Given the difference between the Etest and reference method QC ranges for flucytosine and fluconazole and *C. krusei*, we do not feel that the establishment of even tentative QC limits for these two yeast-antifungal agent combinations is warranted at this time.

In summary, we have provided further documentation of the excellent reproducibility and level of agreement that can be achieved by the Etest methodology for antifungal susceptibility testing of yeasts. Furthermore, we have demonstrated good agreement with the reference macrodilution method and have proposed tentative QC limits for two QC strains and five antifungal agents when tested by the Etest methodology. This information should facilitate further development and broader use of this simple testing method. The convenience of the Etest methodology will allow laboratories to test selectively one or more antifungal agents by using an approach that is now quite familiar to most laboratories involved in antimicrobial susceptibility testing. Additional multicenter studies incorporating a larger number of *Candida* spp. are warranted as we continue to assess the utility of Etest as a means of determining antifungal susceptibility.

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