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Standardized Susceptibility Testing of Fluconazole: an International Collaborative Study

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An international collaborative study of broth dilution (MIC) and disk diffusion susceptibility testing of fluconazole was conducted by using a chemically defined medium (High-Resolution Antifungal Assay Medium; Oxoid Ltd., Basingstoke, United Kingdom) and standard test methods performed in eight reference laboratories. Ten yeast isolates were tested by each test method in duplicate on each of 3 separate days. The intralaboratory reproducibility of the MIC test was excellent; 95.7% of the replicate tests (n = 220) were within 2 doubling dilutions of the other values in the set for the eight laboratories. The intralaboratory reproducibility of the disk test was also good, with 91% of the replicate tests (n = 234) agreeing with each other within an arbitrarily chosen value of 4 mm. Interlaboratory agreement of MIC test results was acceptable, with 84% of the MICs agreeing within 2 doubling dilutions. In contrast, the interlaboratory agreement of the disk test was not good, with only 59% of test results agreeing within 4 mm. Comparison of the rank order of MICs obtained in each laboratory with the reference rank order gave an agreement of 70 to 80% (median, 80%) with the MIC test and 70 to 90% (median, 80%) with the disk test. These preliminary results are encouraging for the development of standardized testing methods for testing fluconazole.

Opportunistic fungal infections are becoming increasingly important causes of morbidity and mortality in hospitalized patients (1, 16, 29). Commensurate with the increase in fungal infections over the past decade has been an increase in the use of systemic antifungal agents worldwide (24) and the introduction of a number of new antifungal agents with systemic activity (4, 12, 21, 23, 25). Among the most notable of the newly introduced antifungal agents is fluconazole, a polar, water-soluble, orally absorbable bistriazole which shares broad-spectrum antifungal activity and a common mechanism of action with other members of the azole group (4, 6, 11, 28). The increased use of new antifungal agents, such as fluconazole, as well as established agents, such as amphotericin B and ketoconazole, has raised some concern regarding the potential for the development of resistance to one or more antifungal agents among clinical isolates of pathogenic fungi (3, 5, 15, 20, 23, 25, 26, 27). As a result, increased attention is now being paid to methods of in vitro susceptibility testing of antifungal agents. Not only are these methods potentially useful in the development and preclinical evaluation of new antifungal agents but clinical laboratories are also being asked to assume a greater role in the selection and monitoring of antifungal therapy for clinical purposes. Because of its potential clinical importance, susceptibility testing of fluconazole is of particular interest and has been the focus of attention for several groups of investigators both in the United States and internationally. Problems with the lack of a standardized test method and little or no established in vivo correlation has caused several investigators to question the value of in vitro susceptibility testing of antifungal agents, particularly fluconazole and other azoles (2, 3, 5, 7, 9, 15, 19, 23).

The collaborative study described here was performed as a preliminary step in the development of standardized broth macrodilution and disk diffusion test methods for fluconazole. The goals of this study were to (i) examine the intra- and interlaboratory reproducibilities of fluconazole MICs and disk test results determined by standardized test methods with a defined medium developed for testing fluconazole and other antifungal agents (High-Resolution Antifungal Assay [HR] Medium; Oxoid Ltd., Basingstoke, United Kingdom) and (ii) assess the ability of the standardized methods to define a rank order of susceptibilities relative to the results obtained with an arbitrarily chosen reference method. We elected to evaluate only the chemically defined HR medium because previous studies have shown that it supports the growth of yeast adequately and that it performs acceptably with other azoles (18). Furthermore, the use of a chemically defined medium avoids the in vitro antagonism of azole

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activity attributed previously to complex undefined media (10, 14, 15).

MATERIALS AND METHODS

Antifungal agents. Fluconazole powder and disks (25-μg disk content) were obtained from Pfizer Central (Sandwich, United Kingdom) from a single lot and were distributed to the participating laboratories. Upon receipt, a concentrated stock solution (12,800 μg/ml) was prepared in dimethyl formamide and frozen at −60°C until used. Fluconazole disks were stored in a desiccator at 4°C.

Test organisms. Nine strains of *Candida* species (five strains of *C. albicans* and one each of *C. krusei*, *C. pseudotropicalis* ["*C. kefyr*"], *C. tropicalis*, and *C. parapsilosis*) and one *Cryptococcus neoformans* strain were selected for testing. The isolates were coded (CA-1 to CA-5, CK-1, CP_S-1, CT-1, CP-1, and C_RN-1, respectively) and were sent to each participating laboratory. These isolates were selected for inclusion in the study on the basis of their widely different susceptibilities to fluconazole, as determined previously by an arbitrarily selected broth macrodilution reference method (23a).

Medium. HR medium was obtained from Pfizer Central Research. HR is a chemically defined medium and has been described previously by Pfaller et al. (18) and Shadomy and Pfaller (22). A single lot of medium was distributed among the participating laboratories, along with detailed preparation instructions. HR medium was buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS) buffer (Sigma, St. Louis, Mo.) for broth macrodilution testing. HR medium was mixed with Oxoid L13 agar No. 3 buffered to pH 7.5 with 0.1 M phosphate buffer for disk testing.

Susceptibility testing procedure. Detailed instructions for performing twofold dilutions, inoculum preparations, and endpoint determinations were provided for each laboratory. Broth macrodilution testing was performed with twofold drug dilutions in HR medium. The stock solution of fluconazole was diluted by previously described methods recommended for minimizing systematic pipetting errors (13). Final fluconazole concentrations were 0.015 to 128 μg/ml.

Yeast inocula were prepared as described previously (17). Briefly, yeasts were grown on Sabouraud dextrose agar for 24 h (*Candida* species) to 48 h (*C. neoformans*) at 28°C, and the inoculum suspension was prepared by picking five colonies of at least 1 mm in diameter and suspending the material in 10 ml of sterile, buffered (pH 7.0; MOPS) HR medium. The yeast suspensions were then incubated overnight (16 h) at 28°C on a rotary shaker (170 rpm). Following incubation, the cell density of each suspension was determined by using a modified Fuchs Rosenthal counting chamber provided to each laboratory. The cell density of each suspension was then adjusted by dilution with buffered HR medium. By this procedure, inocula were 1×10^3 cells per ml for *Candida* species and 5×10^4 cells per ml for *C. neoformans*. In each case, the inoculum size was verified by enumeration of the CFU obtained by subculture on Sabouraud dextrose agar.

Yeast inocula (0.9 ml) were added to sterile, acid-washed, 12-by-75-mm glass tubes containing 0.1-ml aliquots of fluconazole solution (10× final concentration) by using sterile, individual 1-ml pipettes. The contents of the tubes were mixed by inversion. Replicate tests were incubated in air at 28°C, and the loosely capped tubes were inspected 24 and 48 h later. Drug free and yeast free controls were included.

To assess fluconazole activity more precisely, the growth in each tube was scored and recorded as described previously (18), as follows: 0, optically clear; 1+, slightly hazy; 2+, prominent reduction in turbidity compared with that of the drug-free control; 3+, slight reduction in turbidity compared with that of the drug-free control; 4+, no reduction in turbidity compared with that of the drug-free control. On the basis of this scoring system, the MIC was defined as the lowest fluconazole concentration in which the growth score was 1+ or less.

Disk diffusion testing was performed by adjusting the yeast inoculum suspension, which was prepared as described above, to an initial density of 4×10^7 cells per ml and adding 0.08 ml of the suspension to 10 ml of molten HR medium (final inoculum, 3×10^5 cells per ml of agar). The agar suspension was poured into 9-cm-diameter petri dishes and allowed to harden. A 25-μg fluconazole disk was placed onto the center of the agar, and the plates were incubated in air at 28°C for 24 h. Zones of inhibition were viewed on a light box, and the diameters were measured along two axes at right angles, using metric vernier calipers. This approach provided two readings for each disk test, with all isolates being tested in duplicate on each of 3 different days (total of 12 datum points for each isolate).

Study design and analysis of results. Eight laboratories (referred to as laboratories A to F, H, and P [see Table 1]) participated in the study. Each laboratory received individual subcultures of the test isolates, each of which was identified by a coded number. Intralaboratory variation was assessed by testing each isolate in duplicate on each of 3 separate days. Results were recorded on data sheets supplied to each laboratory and were submitted to a coordinating laboratory for analysis. For the MICs, the differences among replicates were considered acceptable if they were within fourfold of the other values in their set (18). For the disk test, an arbitrary value of a ≤4-mm difference in zone diameters among replicates was selected as the limit of acceptable variation.

The median value from each laboratory for each isolate was determined from the replicate MICs and disk test results (zone diameters). These median values were used to examine agreement among laboratories and to determine the rank order of susceptibility of isolates to fluconazole for each laboratory. The analysis of the MIC data included both MICs for which endpoints were obtained (on-scale results) and those which were off scale (>128 μg/ml). For purposes of analysis, when an MIC was greater than the highest fluconazole concentration used (i.e., >128 μg/ml), it was assigned an endpoint value of 256 μg/ml. Isolates were ranked according to their relative susceptibilities (low to high), and the resulting rank order was compared with that established by the reference broth macrodilution method in previous studies (23a).

RESULTS

Variability of replicates within laboratories. Differences among replicates tested within each laboratory were used to estimate intralaboratory variability. Overall, 220 replicate sets of MICs were evaluable and contained 440 datum points, of which 421 (95.7%) were within fourfold of the others in their set (Table 1). Intralaboratory reproducibility was ≥98% for six of the eight laboratories that provided such data. Eighty percent of the discrepant sets were observed in two of the participating laboratories. The intralaboratory reproducibility of the disk test was also good. Overall, 234 replicate sets of disk test results were evaluable and contained 930 datum points, of which 848 (91%) were within 4

TABLE 1. Reproducibility of fluconazole MICs and disk test results within each laboratory[a]

Strain[b]	Test method	% in laboratory:							
		A	B	C	D	E	F	H	P
CA-1	MIC	100	100	100	100	100	100	67	100
	Disk	92	75	67	83	100	100	67	100
CA-2	MIC	100	100	100	100	100	83	67	100
	Disk	100	67	100	67	100	100	83	100
CA-3	MIC	100	100	100	100	100	100	100	100
	Disk	100	100	100	100	100	83	100	100
CA-4	MIC	100	100	100	100	100	100	100	100
	Disk	100	100	100	83	100	100	100	100
CA-5	MIC	100	67	100	100	100	100	100	100
	Disk	100	67	100	100	100	100	100	92
CK-1	MIC	100	100	100	100	100	100	100	100
	Disk	92	100	100	83	100	92	100	100
CP-1	MIC	100	67	100	100	100	100	67	100
	Disk	100	75	100	92	100	100	75	100
CP_S-1	MIC	100	100	100	100	100	100	67	100
	Disk	100	80	100	58			67	100
CT-1	MIC	100	100	100	100	100	100	50	83
	Disk	92	92	92	33	100	100	83	75
C_RN-1	MIC	100	67	100	100	100	100	100	100
	Disk	83	88	83	83	100	67	100	100
Overall	MIC[c]	100	90	100	100	100	98	82	98
	Disk[d]	96	84	94	78	100	94	88	97

[a] Reproducibility indicated as the percentage of replicate MICs within fourfold or the percentage of replicate disk tests within 4 mm in each laboratory.

[b] Abbreviations: CA-1 through CA-5; *C. albicans* 1 through 5, respectively; CK-1, *C. krusei* 1; CP-1, *C. parapsilosis* 1; CP_S-1, *C. pseudotropicalis* (kefyr) 1; CT-1, *C. tropicalis* 1; C_RN-1, *C. neoformans* 1.

[c] Values are for 220 replicate sets of MICs (440 datum points). The overall reproducibility for all laboratories and all isolates was 95.7%.

[d] Values are for 234 replicate sets of disk tests (930 datum points). The overall reproducibility for all laboratories and all isolates was 91%.

mm of others in their set (Table 1). Intralaboratory reproducibility was ≥94% for five of the eight laboratories that provided disk test results. Seventy-two percent of the discrepant values were observed in three of the participating laboratories.

Variability among laboratories. The levels of interlaboratory agreement of MIC results stratified by test strain are given in Table 2. Overall, 84% of the endpoint readings varied fourfold or less among the eight participating laboratories. The highest level of agreement (100% within fourfold of the others in their set) was observed with strains CA-1 to CA-4 and CK-1. Seventy-five percent of the endpoint readings for strains CP-1, CP_S-1, and C_RN-1 varied fourfold or less among the eight laboratories. Of the six outlying MICs, five (83%) were off by only one to two tubes and one was 16- to 32-fold lower than the other MICs for the test strain (C_RN-1). In contrast, CA-5 (50% agreement) and CT-1 (63% agreement) caused significant problems, with results varying by more than 32-fold among the participating laboratories (Table 2). By comparison with the MIC results, the interlaboratory agreement observed with the disk test was poor (Table 2). Only 59% of the disk test results among the eight participating laboratories agreed within 4 mm. The highest level of agreement was only 88%, which was observed with strain CA-4, and the agreement was >70% for only one additional isolate (CA-3 [75%]). In general, the level of agreement was lowest for those isolates with larger inhibition zones (>25 mm). As with the MIC test, isolate CT-1 caused significant problems, with an agreement of only 38%.

TABLE 2. Interlaboratory agreement of fluconazole MIC and disk test results

Strain[a]	% of MIC readings that varied fourfold or less	% of disk test results within 4 mm
CA-1	100	50
CA-2	100	63
CA-3	100	75
CA-4	100	88
CA-5	50	63
CK-1	100	63
CP-1	75	50
CP_S-1	75	67
CT-1	63	38
C_RN-1	75	38
Overall	84	59

[a] See note *b* of Table 1.

TABLE 3. Agreement of fluconazole susceptibility test results with the reference rank order

Reference rank order[a]	Reference MIC (μg/ml)	Median values from all laboratories	
		MIC (μg/ml)	Zone (mm)
CA-1	1.5	2	35
CA-2	2.0	1	36
CP_S-1	4.0	1	50
CP-1	8.0	7	45
C_RN-1	16	32	27.5
CT-1	16	160	31.5
CA-5	48	40	22.5
CA-4	64	64	22
CK-1	64	96	17.5
CA-3	128	96	15

[a] See note *b* of Table 1.

Relative susceptibilities of isolates. To assess the extent to which laboratories identified a similar relative susceptibility pattern among isolates, the rank order of susceptibilities of the test isolates, as determined by MIC and disk testing in each laboratory, was compared with the reference rank order (Table 3).

The overall rank order of susceptibility, as determined by the median MIC for all tests in all laboratories, was in close agreement with the reference rank order with the exception of that for isolate CT-1 (Table 3). CT-1, which was classified as moderately susceptible (MIC, 16 μg/ml) by the reference laboratory, was misclassified as either the most resistant or the second most resistant by five of the eight laboratories. With the exception of CT-1, and possibly C_RN-1 and CA-5, the laboratories were able to correctly classify the four most susceptible (MIC, ≤8 μg/ml) isolates (CA-1, CA-2, CP_S-1, and CP-1) and the three most resistant (MIC, >32 μg/ml) isolates (CA-4, CK-1, and CA-3) as such. The agreement with the reference rank order obtained with the MIC test ranged from 70 to 80% (median, 80%) among the participating laboratories.

The overall rank order of susceptibility, as determined by the disk test zone diameters, was also in agreement with the reference rank order (Table 3). The laboratories were able to correctly classify the four most susceptible (zone diameters, ≥35 mm) isolates (CA-1, CA-2, CP_S-1, and CP-1) and the four most resistant (zone diameters, ≤23 mm) isolates (CA-5, CA-4, CK-1, and CA-3) as such. Again, the moderately susceptible isolates, CT-1 and C_RN-1, were proble-

matic with the disk test, as they were with the MIC test. The agreement with the reference rank order obtained with the disk test ranged from 70 to 90% (median, 80%) among the participating laboratories.

DISCUSSION

The major sources of susceptibility test variation for fluconazole and other azoles in vitro have been reported to be the pH, the composition of the test medium, inoculum size, temperature, and duration of incubation (8, 15, 22). In addition, partial inhibition of fungal growth in vitro often takes place over a range of fluconazole concentrations, which can make endpoint determinations both difficult and subjective (15, 22). In the present study, we attempted to control these variables by using standard protocols for broth macrodilution and disk diffusion testing of fluconazole and examined intra- and interlaboratory reproducibilities as well as rank order agreements within a panel of yeast isolates. For the MIC test method, we used a chemically defined, buffered (pH 7.0; MOPS) medium, an inoculum size of 1×10^3 cells per ml (5×10^4 cells per ml for *C. neoformans*), and incubation conditions of 28°C for 48 h. The problem of endpoint determination was addressed by applying a standard scoring system described previously by Pfaller et al. (18). This method was similar to that described by Shadomy and Pfaller (22) and Pfaller et al. (18); however, it differed in the method of preparing the inoculum (hemacytometer count versus spectrophotometer) and in the incubation temperature (28 versus 35°C). The use of a hemacytometer and incubation at 28°C were selected in order to accommodate all participating laboratories. The actual MICs may be influenced greatly by differences in inoculum preparation and incubation temperature; however, since the goal of this study was to compare results among laboratories by using a common protocol, we chose to narrow our focus and examine only a single set of parameters. We did not attempt to compare fluconazole MIC results obtained with the present protocol with the results obtained by adhering strictly to the protocol of Pfaller et al. (18).

An essential requirement of a medium selected for antimicrobial susceptibility testing is that it must support adequate growth of the test organisms. Previous studies have documented the ability of HR medium to support the growth of *Candida* species (18). Although HR and other chemically defined media can support the growth of *C. neoformans*, this organism grows more slowly than *Candida* species in defined medium, and thus, a higher starting inoculum is required for *C. neoformans* to allow for endpoint determination within the allotted 48-h incubation time.

The intralaboratory reproducibilities of fluconazole MIC and disk test results were 95.7 and 91%, respectively. Although these are the first such data reported for fluconazole, these findings compare favorably with the 97% intralaboratory reproducibility reported by Pfaller et al. (18) for other antifungal agents and offer further proof that variation in in vitro test results among laboratories cannot be attributed to intralaboratory errors (2, 8, 18). It should be noted that in contrast to the study of Pfaller et al. (18), the isolates in the present study were coded but the investigators were not blinded since the codes were easily recognized (e.g., CA-1 for *C. albicans* 1). Thus, the isolates used in the duplicate and triplicate repeat tests all had the same code number and were known to be the same by each investigator.

The level of interlaboratory agreement of MIC results was considerably better than that reported for other antifungal agents by nonstandardized methods (2, 8) and was comparable to that of the previous study of Pfaller et al. (18) (84% overall agreement with HR medium) (18). Although interlaboratory agreement was 75 to 100% for 8 of the 10 test isolates, considerable problems were encountered with isolates CT-1 (63%) and CA-5 (50%). The reasons for the discrepancies observed among laboratories with these two isolates are not readily apparent. The specific growth characteristics of these strains may have contributed to difficulties in defining the endpoints in certain laboratories; however, they were not investigated further in this study. Some isolates give sharper endpoints than others in testing the activity of fluconazole. Whether this is purely a methodologic problem or has some biologic and/or clinical significance is unknown and awaits further study.

In contrast to the agreement observed with the MIC test, the level of agreement among laboratories with the disk test was not good (Table 2). This is most likely due to the rather diffuse zone margins observed with most of the test isolates. Members of each laboratory used their own judgments and experiences in determining the zone margins, and although the laboratories were internally consistent, there was no attempt in this study at standardization of this measurement among laboratories. Although further refinement of the disk test may sharpen the zone margins, the interlaboratory variability may be improved by the development of quality control strains with well-defined disk test zone diameters that serve as standards.

In agreement with the previous studies of Calhoun et al. (2), Galgiani et al. (8), and Pfaller et al. (18), the rank order of MIC and disk test results was found to be similar among the participating laboratories and was generally in agreement with the reference rank order. The exception to the otherwise excellent rank order agreement was strain CT-1, which was misclassified by the MIC test as highly resistant in five of the eight laboratories.

In summary, the preliminary results of this international collaborative investigation indicate that broth macrodilution susceptibility testing with fluconazole, when performed by the protocol used in this study, provides acceptable intralaboratory agreement. The interlaboratory agreement (84%) appeared to be acceptable overall; however, the extreme variability observed with strains CT-1 and CA-5 and the modest agreement (75%) observed with strains CP-1, CP_S-1, and C_RN-1 suggest that certain strains of yeast may pose a problem for the method and that additional studies will be necessary to develop an optimal MIC test method. Despite these reservations, the rank order of susceptibilities determined in the participating laboratories was similar to the reference rank order. The MIC endpoints spanned a wide range (0.5 to >128 μg/ml) and were determined easily by using the scoring system described in the protocol. Although the disk test was internally reproducible and provided results that were in general agreement with the reference rank order, the low interlaboratory reproducibility indicates that additional work is necessary before the disk test can be considered a reliable means of performing fluconazole susceptibility testing. Such studies are under way. Despite this progress in developing standardized susceptibility testing methods, the clinical significance of fluconazole susceptibility testing remains unclear, and routine testing for purposes of clinical decision making is not justified unless there is concern because of an apparent lack of clinical response to therapy.

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