JOURNAL OF CLINICAL MICROBIOLOGY, Aug. 1988, p. 1437–1441
0095-1137/88/081437-05$02.00/0

Vol. 26, No. 8

Multicenter Evaluation of Four Methods of Yeast Inoculum Preparation

MICHAEL A. PFALLER,[1,2]* LEON BURMEISTER,[3] MARILYN S. BARTLETT,[4] AND MICHAEL G. RINALDI[5]

Veterans Administration Medical Center[1] and Departments of Pathology[2] and Preventive Medicine,[3] University of Iowa College of Medicine, Iowa City, Iowa 52242; Department of Pathology, Indiana University School of Medicine, Indianapolis, Indiana 46223[4]; and Veterans Administration Medical Center and Department of Pathology, Texas Health Science Center, San Antonio, Texas 78284[5]

Received 8 February 1988/Accepted 14 April 1988

We initiated a comparative study of four methods of yeast inoculum preparation: a spectrophotometric method, the Wickerham card method, a hemacytometer method, and the Prompt inoculation system. The variability in inoculum size obtained when each method was applied to two strains each of *Candida albicans*, *Candida tropicalis*, *Candida parapsilosis*, *Torulopsis glabrata*, *Cryptococcus neoformans*, and *Saccharomyces cerevisiae* was analyzed in a single laboratory. Each method was performed in triplicate on the same day and on three separate days to provide estimates of within-day and between-day variations. Inoculum size was determined by viable colony counts. The greatest range of inoculum sizes was seen with the Wickerham card method. Viable counts ranged from 1.1×10^6 to 24.2×10^6 CFU/ml among the 12 yeast isolates. The greatest variation was observed with the Prompt system. Within-day coefficients of variation averaged 19% (range, 4 to 45%), and between-day coefficients of variation averaged 22% (range, 3 to 51%). Variation between laboratories was evaluated by comparing inoculum values obtained by each method in three different laboratories for two strains of *C. albicans*. The spectrophotometric method was the least variable and the Wickerham card and hemacytometer methods were the most variable methods between laboratories. The spectrophotometric method is recommended as the method of choice for preparation of a standardized inoculum suspension for susceptibility testing of yeasts.

The performance of antifungal susceptibility testing of clinical yeast and yeastlike fungal pathogens is becoming increasingly important in directing the therapy of serious fungal infections (1, 4, 16, 19). Unfortunately, the methods for performing these tests are nonstandardized (2, 5, 6). As noted previously for bacterial susceptibility testing (11, 18), variation in key technical steps such as inoculum preparation, medium composition, pH, length of incubation, and method of endpoint reading have all been cited as reasons for variability in test results within a given laboratory and between laboratories (1, 2, 5–7, 12). By analogy to standardization of bacterial susceptibility testing, it has been suggested that analysis and standardization of each test condition might increase the reproducibility of the broth dilution method for fungal susceptibility testing (5, 12).

One of the most important steps in performing antifungal susceptibility testing of clinical yeast isolates is inoculum preparation (5, 7, 14). Several methods of inoculum preparation have been recommended, including the spectrophotometric (1, 17), Wickerham card (17), and hemacytometer count (9, 10) methods. Although not specifically developed for yeast inoculum preparation, the commercially available Prompt inoculation system (3M, St. Paul, Minn.) provides an additional technique which has been shown to produce a reproducible, standardized bacterial inoculum (8) and has also been shown to be adaptable to yeast inoculum preparation (14). The present study was undertaken to determine the day-to-day and between-laboratory variabilities in inocula prepared by each of these techniques. These efforts will allow selection of one method of inoculum preparation for use in developing a standardized susceptibility testing method.

* Corresponding author.

MATERIALS AND METHODS

Study design. Three laboratories participated in the study and will be referred to subsequently as laboratories 1, 2, and 3. A panel of clinical yeast and yeastlike isolates (described below) was prepared and distributed by Smith Shadomy, Medical College of Virginia. Inoculum suspensions of each isolate were prepared in laboratory 1 as described below, by using the spectrophotometric method, the Wickerham card method, the hemacytometer count method, and the Prompt inoculation system. Each method was performed in triplicate on the same day and on three separate days to provide estimates of within-day and between-day variations. Laboratories 2 and 3 prepared inoculum suspensions with each method on three separate days, using *Candida albicans* isolates only. The final inoculum size in each case was quantitated by determining viable colony counts. A common-lot barium sulfate turbidity standard (0.5 McFarland; Difco Laboratories, Detroit, Mich.) and a killed *C. albicans* blastospore suspension (5×10^6 organisms per ml in Formalin) were tested at intervals in each laboratory to provide comparative data on spectrophotometer performance and hemacytometer counting.

Test organisms. The panel of test organisms developed for the study were well-characterized isolates and included two strains of each as follows: *C. albicans* 7.349 and 7.363, *Candida tropicalis* 52.304 and 52.397, *Candida parapsilosis* 52.356 and 52.408, *Torulopsis glabrata* 49.70 and 49.75, *Cryptococcus neoformans* 9.803 and 9.812, and *Saccharomyces cerevisiae* 35.12 and 35.13. In each case, the

TABLE 1. Comparative data on spectrophotometer performance and hemacytometer counting[a]

Laboratory	Results with:	
	Spectrophotometer (*n*)[b]	Hemacytometer (*n*)[c]
1	73 ± 4 (31)	6.4 ± 0.8 (71)
2	79 ± 4 (15)	6.0 ± 1 (6)
3	80 ± 4 (12)	1.1 ± 0.1 (6)

[a] Spectrophotometer readings were performed on common-lot barium sulfate standard, and hemacytometer counts were performed on a killed *C. albicans* suspension provided to each laboratory.
[b] Values are mean percent turbidity at 530 nm ± standard deviation.
[c] Values are mean number of cells × 10^6 per milliliter ± standard deviation.

accession numbers refer to the Shadomy collection. The organisms were stored on agar slants at room temperature until used in the study.

Method of inoculum preparation. All organisms were subcultured from stock vials onto Sabouraud dextrose agar and passaged at least twice to ensure viability. Incubation conditions throughout were 30°C in air, as recommended in the *Manual of Clinical Microbiology* (17). For methods other than the Prompt inoculation system, the inoculum was prepared by picking five colonies of ≥1 mm in diameter from 48-h-old cultures and suspending the material in 5 ml of sterile 0.85% saline. The resulting suspension was vortexed for 15 s, and the cell density was adjusted by the methods of inoculum preparation described below.

Spectrophotometric method. The turbidity of the cell suspension measured at 530 nm, as described in the *Manual of Clinical Microbiology* (17), was adjusted to match that of the common-lot turbidity standard by using sterile saline as needed.

Wickerham card method. As described in the *Manual of Clinical Microbiology* (17), the suspension to be adjusted was diluted into a tube of sterile 0.85% saline, which was placed against a card bearing several sharply ruled lines. For purposes of the study, identical cards were prepared and distributed to each laboratory. The desired endpoint was reached when there was obvious turbidity but the lines were still sharply defined when viewed through the suspension. The turbidity was approximately the same as the common-lot turbidity standard (0.5 McFarland) which was used for comparison.

Hemacytometer method. By using a standard hemacytometer (American Optical Corp., Buffalo, N.Y.), the concentration of cells in the suspension was determined and the suspension was diluted with sterile 0.85% saline to achieve a cell concentration of 3 × 10^6 to 5 × 10^6 organisms/ml. The actual cell count obtained after dilution was confirmed by performing a cell count on the diluted suspension. The procedure for performing the cell count was that outlined by Nelson (13). By convention, all budding and clumped cells were counted as one cell when this method was used.

Prompt inoculation system. The commercially available Prompt inoculation system uses a grooved plastic rod (inoculation wand) to pick up and release a specific number of organisms from a primary culture plate and thus provide an inoculum without incubation or conventional turbidity adjustment (8, 14, 20). The Prompt systems were obtained from the manufacturer and consisted of an inoculation wand and a plastic inoculum tube (no. 6306) containing 1.15 ml of 0.85% saline. When the Prompt system was used to prepare a yeast cell suspension, a total of five colonies of ≥1 mm in diameter were picked from 24- to 48-h-old cultures by using the disposable inoculation wand and were placed into the inoculum tube. The unit was vortexed for 15 s to release the cells from the grooves of the inoculation wand and to obtain a homogeneous suspension. The suspension was used immediately without incubation or turbidity adjustment.

Inoculum quantitation. Inoculum quantitation was done for each method by quantitative plating on Sabouraud dextrose agar to determine viable cell counts. Five serial 10-fold dilutions of each inoculum suspension were performed in sterile distilled water, and a 10 μl portion of each dilution was spread over the surface of the agar plates with a sterile glass rod. The plates were incubated for 48 h at 30°C in air, and colonies were counted with an electric colony counter (American Optical).

Statistical analysis. The data were analyzed by nonparametric one-way and two-way analyses of variance. Differences between the coefficients of variation (CVs) for the different test methods within one laboratory and among the different laboratories were assessed by the Tukey Student-ized range test. Comparisons were considered significant at the 0.05 level (3, 15).

RESULTS

Spectrophotometer performance and hemacytometer counting. The results of the spectrophotometer readings of the turbidity standard and the hemacytometer counts performed on the killed *C. albicans* for each laboratory are presented in Table 1. The values obtained with the two standards by each technique were highly consistent within each laboratory and, with the exception of significantly lower hemacytometer

TABLE 2. Within-day variation of inoculum values obtained with four methods and several organisms in a single laboratory

Organism and strain	10^6 CFU/ml (% CV) by:			
	Spectrophotometer	Wickerham card	Hemacytometer	Prompt
Candida albicans 7.349	3.7 (14)	6.5 (13)	2.2 (9)	4.2 (7)
C. albicans 7.363	3.1 (4)	4.7 (10)	1.8 (26)	1.2 (43)
C. tropicalis 52.304	3.6 (18)	3.8 (7)	1.7 (40)	1.6 (4)
C. tropicalis 52.397	2.2 (4)	6.9 (10)	3.1 (20)	0.8 (15)
C. parapsilosis 52.356	5.1 (2)	10.0 (18)	1.9 (12)	1.6 (45)
C. parapsilosis 52.408	3.8 (7)	12.8 (10)	1.7 (5)	4.1 (9)
Torulopsis glabrata 49.70	4.0 (4)	13.4 (13)	1.2 (21)	0.2 (28)
T. glabrata 49.75	9.9 (4)	24.2 (19)	2.0 (9)	7.6 (20)
Cryptococcus neoformans 9.803	5.0 (4)	1.1 (9)	2.1 (12)	3.4 (12)
C. neoformans 9.812	4.4 (6)	12.1 (14)	2.1 (5)	2.7 (17)
Saccharomyces cerevisiae 35.12	2.7 (8)	5.4 (7)	2.0 (6)	1.5 (19)
S. cerevisiae 35.13	3.4 (1)	1.2 (3)	2.4 (9)	3.0 (8)

TABLE 3. Between-day variation of inoculum values obtained with four methods and several organisms in a single laboratory

Organism and strain	10^6 CFU/ml (% CV) by:			
	Spectrophotometer	Wickerham card	Hemacytometer	Prompt
Candida albicans 7.349	4.0 (18)	9.4 (34)	2.0 (10)	4.0 (17)
C. albicans 7.363	2.8 (9)	4.0 (31)	1.9 (24)	1.8 (45)
C. tropicalis 52.304	2.8 (22)	4.5 (13)	2.4 (9)	1.6 (12)
C. tropicalis 52.397	2.3 (17)	6.0 (10)	2.4 (3)	1.0 (25)
C. parapsilosis 52.356	5.1 (9)	12.9 (26)	2.1 (1)	2.1 (20)
C. parapsilosis 52.408	3.9 (4)	18.5 (60)	1.8 (6)	4.4 (10
Torulopsis glabrata 49.70	4.3 (5)	15.9 (15)	1.1 (25)	0.4 (51)
T. glabrata 49.75	9.5 (7)	39.2 (13)	2.4 (15)	8.8 (3)
Cryptococcus neoformans 9.803	5.3 (2)	1.2 (4)	2.2 (13)	3.9 (17)
C. neoformans 9.812	4.6 (10)	11.6 (8)	2.5 (9)	3.3 (21)
Saccharomyces cerevisiae 35.12	2.9 (15)	5.4 (8)	2.1 (3)	1.3 (25)
S. cerevisiae 35.13	3.3 (2)	1.1 (14)	1.9 (29)	3.4 (16)

counts obtained from laboratory 3 with the killed *C. albicans* suspension ($P < 0.05$ versus laboratories 1 and 2), were generally comparable among the three participating laboratories.

Inoculum values and variation observed in a single laboratory with various yeast isolates. With the exception of the Wickerham card method, the inoculum size obtained by the various methods of inoculum preparation in laboratory 1 did not vary appreciably among the different species of isolates tested (Table 2). In general, the Wickerham card method produced the largest inoculum size, regardless of the test organism employed. The greatest degree of within-day variation was observed with the Prompt system, with a mean CV of 19%, and the least variation was observed the spectrophotometric method, with a mean CV of 6% (comparison between spectrophotometric and Prompt methods significant at the 0.05 level).

Both the Prompt inoculation system and the Wickerham card method showed considerable between-day variation, with mean CVs of 22 and 20%, respectively (Table 3). The Prompt method had the highest CV of the four methods in 50% of the isolates tested. Both the spectrophotometric and the hemacytometer methods demonstrated comparable between-day reproducibility, with mean CVs of 10 and 12, respectively. Of the four methods evaluated for between-day reproducibility, only the comparison between the spectrophotometric and the Prompt methods was significantly different at the 0.05 level.

Inoculum values and variation observed within and between multiple laboratories. The highest inoculum size and greatest between-day variability in each of the participating laboratories were observed with the Prompt and Wickerham card methods (Tables 2 to 4). The rank order of the methods according to variation indicated that the Wickerham card method was the most variable method of inoculum preparation in all three laboratories. The spectrophotometric method was the least variable in two of the three laboratories; however, there were no significant differences in the variability observed with this method versus that with the other methods ($P > 0.05$ for all comparisons).

Although in each case the range of inoculum values obtained from the three laboratories was considerable (Table 5), there was no significant difference in the mean inoculum size obtained with each of the four methods of inoculum preparation ($P > 0.05$ for all comparisons). The rank order of the methods according to CV indicated that the method with the greatest variation between laboratories was the hemacytometer count method, followed closely by the Wickerham card method. Although the spectrophotometric method was the least variable of the four methods between laboratories (rank order), the observed differences in interlaboratory variation were not statistically significant ($P > 0.05$ for all comparisons).

DISCUSSION

In general, with the exception of the Wickerham card method, inoculum suspensions of 1×10^6 to 5×10^6 CFU/ml were reliably prepared for most species with each method of inoculum preparation by picking at least five colonies of ≥ 1 mm in diameter, although more-concentrated suspensions were observed with one strain of *T. glabrata*, which pro-

TABLE 4. Between-day variation of inoculum values of *C. albicans* obtained with four methods in each of three laboratories

Laboratory and *C. albicans* strain	10^6 CFU/ml (% CV) by:			
	Spectrophotometry	Wickerham card	Hemacytometer	Prompt
1				
7.349	4.0 (18)	9.4 (34)	2.0 (10)	4.0 (17)
7.363	2.8 (9)	4.0 (31)	1.9 (24)	1.8 (45)
2				
7.349	3.0 (26)	4.2 (79)	1.3 (31)	7.9 (44)
7.363	1.7 (31)	2.9 (31)	1.4 (26)	5.8 (40)
3				
7.349	1.6 (21)	0.6 (21)	6.1 (21)	8.7 (8)
7.363	1.4 (32)	0.8 (41)	6.9 (28)	8.5 (17)

TABLE 5. Between-laboratory variation of inoculum values of *C. albicans* obtained with four methods[a]

Method	Results with strain: 7.349			7.363		
	Range[b]	Mean[b]	CV (%)	Range[b]	Mean[b]	CV (%)
Spectrophotometry	1.6–4.0	2.8	42	1.4–2.8	1.9	38
Wickerham card	0.6–9.4	4.8	93	0.8–4.0	2.6	63
Hemacytometer	1.3–6.1	3.1	83	1.4–6.9	3.4	89
Prompt	4.0–8.7	6.9	37	1.8–9.5	5.7	67

[a] Values obtained from three laboratories. The value for each method represents the mean of three separate determinations.
[b] Viable CFU $\times 10^6$ per milliliter.

duced smaller yeasts. This inoculum density is sufficient, following appropriate dilution, for the performance of broth dilution susceptibility testing, in which a final inoculum of between 5×10^4 and 5×10^5 CFU/ml is required (10).

The degrees of variability observed with the Wickerham card method and the Prompt inoculation system between days within a given laboratory and between laboratories in the present study indicate that these methods are too variable to be recommended for standardized inoculum preparation. Although the hemacytometer count method was very reproducible within any given laboratory, it was the most variable of the four methods when between-laboratory variation was assessed (Table 5). This is further supported by the data presented in Table 1, which shows that the counts obtained with the killed *C. albicans* suspension in laboratory 3 were significantly different from those obtained in laboratories 1 and 2 ($P < 0.05$). Thus, despite the objectivity of the hemacytometer count method, it is not the optimal method for the preparation of a standardized inoculum suspension.

Throughout the study, the most reproducible method of inoculum preparation, both within a given laboratory and between laboratories, was the spectrophotometric method. This method was simple and objective and was not significantly affected by the genus or species tested. The availability of stable turbidity standards provides an additional means of standardization and quality control for this method that is not readily achieved with the other methods of inoculum preparation.

In summary, all four methods of inoculum preparation included in this study exhibited some variability within a given laboratory and between laboratories. Although it is difficult to say how much variability in the inoculum is acceptable, it was clear in the present study that the spectrophotometric method was consistently the least variable of the four methods evaluated. On the basis of these findings, we recommend that the spectrophotometric method be considered the method of choice for the preparation of a standardized yeast inoculum suspension. Subsequent efforts in developing a standardized method of antifungal susceptibility testing will provide additional opportunities to evaluate the feasibility of this approach and additional recommendations concerning the specific inoculum concentration to be used in testing the various antifungal agents.

ACKNOWLEDGMENTS

This work was supported in part by the Veterans Administration and by a grant from Miles Laboratories.

We acknowledge the helpful comments and suggestions of John N. Galgiani and the members and advisors of the National Committee for Clinical Laboratory Standards Subcommittee on Antifungal Susceptibility Testing. The technical expertise of the technologists and research assistants from the participating laboratories was greatly appreciated. We are particularly grateful to Difco Laboratories and 3M for supplying the turbidity standards and the Prompt inoculation systems, respectively, for use in this study.

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