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Modifications of a *Candida albicans* Biotyping System

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Methods of preparing and plating inocula onto media for the Odds and Abbott *Candida albicans* biotyping system (F. C. Odds and A. B. Abbott, Sabouraudia 18:301–317, 1980; Odds and Abbott, Sabouraudia 21:79–81, 1983) were altered to utilize inexpensive and commercially available supplies and equipment. The modified system correlates well with the reference system.

Candida albicans is an opportunistic pathogen which commonly infects immunocompromised hosts (1–6,11). Recently, with the development of systems to differentiate among *C. albicans* strains (7, 9, 12, 13), epidemiological studies have become possible as a means to understand such opportunistic infections. The *C. albicans* biotyping system described by Odds and Abbott (7) is an excellent differentiation system which unfortunately has some limitations. Specifically, both the methods of preparing yeast cell suspensions and inoculating the fungi onto the agar require the use of custom-built devices which makes the system costly and inconvenient. Our laboratory has developed alternatives for both the preparation of inocula and the inoculation method using commercially available materials, resulting in greater convenience and reduction of the cost. This paper not only discusses these modifications but also shows the reproducibility of these altered techniques.

The Odds and Abbott *C. albicans* biotyping system consists of assessing the growth of the test strain on nine agar plates of various biochemical compositions. This system determines pH 1.4 and salt tolerance; flucytosine, borate, and safranine resistance; production of proteinase; and assimilation of urea, sorbose, and citrate. The nine biochemical tests are grouped in sets containing three tests each. A positive result for each test is assigned a specific numerical value. The summation of these values from each set results in a 3-digit biotype number (5, 7, 8). For the Odds and Abbott system, preparation of inocula to a concentration of 5.0×10^5 to 5.0×10^6 yeast cells per ml is needed for inoculation onto test media (7). Odds and Abbott used a custom-built device to adjust the fungal concentration to this range. We explored two readily available alternate means for obtaining these fungal concentrations: the Klett-Summerson photoelectric colorimeter (Klett Manufacturing Co., Inc., New York, N.Y.) and McFarland standards (Scott Laboratories, Inc., Carson, Calif.).

To determine the reproducibility of these alternative methods, five reference strains (*C. albicans* 73/005 and 73/026, *C. tropicalis* 73/071 and 75/043, and *C. krusei* 74/041) described in the original paper of Odds and Abbott (7) and 13 subsequent reference strains (*C. albicans* M3960, 26A, 265f, 50A, and Sumner; *C. guilliermondii* D52/D, 83F430N, and Hosoason; *C. glabrata* J. Hughes and SH2/T; *C. parapsilosis* X4222A and 85/015C; and *Saccharomyes cerevisiae* X3350), all graciously supplied by F. C. Odds, were repeatedly tested in these systems. Each isolate was maintained by monthly transfers to Sabouraud dextrose agar plates and was stored at 4°C. Before preparation of inocula, each isolate was grown on a fresh Sabouraud plate. One colony from each 24- to 72-h-old plate was then streaked onto a plate containing yeast nitrogen base glucose (7). After the isolates were incubated at 37°C for 24 to 30 h, yeast cells were removed with a cotton swab and suspended in 5 ml of sterile distilled water. First, using the Klett colorimeter, the yeast cell density was adjusted to 95 Klett units. Since 95 Klett units is equivalent to approximately 10^7 cells per ml for most yeasts and the desired suspension for inoculation onto agar plate surfaces ranges from 5.0×10^5 to 5.0×10^6, each suspension was subsequently diluted 1:10. Quantitative plate counts were performed for each of the 18 reference strains. The mean CFU per milliliter ($n = 63$) ranged from 4.4×10^5 to 3.2×10^6, concentrations very close to the recommended range for use in the biotyping system.

Secondly, the use of McFarland standards was analyzed as an additional alternative for establishing fungal concentrations. To determine the reproducibility of this second method, 6 of the 18 reference strains, 73/005, 73/026, 73/071, 74/041, 75/043, and D52/D, were grown as described above and suspended in 5.0 ml of distilled water. These six strains were specifically chosen because they represented differences in degree of granularity when suspended. It was determined that McFarland standard 1.0 has a density of approximately 95 Klett units. Four different laboratory personnel adjusted the density of the six strains by using McFarland standard 1.0 as a guide for the ideal concentration. To use this standard for adjusting cell density, a dark background against which the cell concentrations were read and two additional McFarland standards, 0.5 and 3.0, for comparison purposes in adjusting the cell density were used. By using this McFarland standard method of adjusting cell density and then diluting 1:10, quantitative plate counts showed the actual range of CFU per milliliter to be 5.0×10^5 to 2.5×10^6, values all within the ideal inoculum range for the Odds and Abbott system. Hence, either the Klett colorimeter or McFarland standards could substitute for the Odds and Abbott custom-built devices in establishing appropriate inoculum concentrations for the biotyping system.

In addition to modifications of inoculum preparation, the method of inoculating these cells onto agar plates was altered. For the original biotyping system, a specially designed and again custom-built multipronged inoculator was used. In our laboratory, a commercially available device (Clonemaster Manifold Dispenser; Immunsine Laboratories, Berkeley, Calif.) was substituted for this multipronged device. The Clonemaster contains eight rows of 12 prongs each

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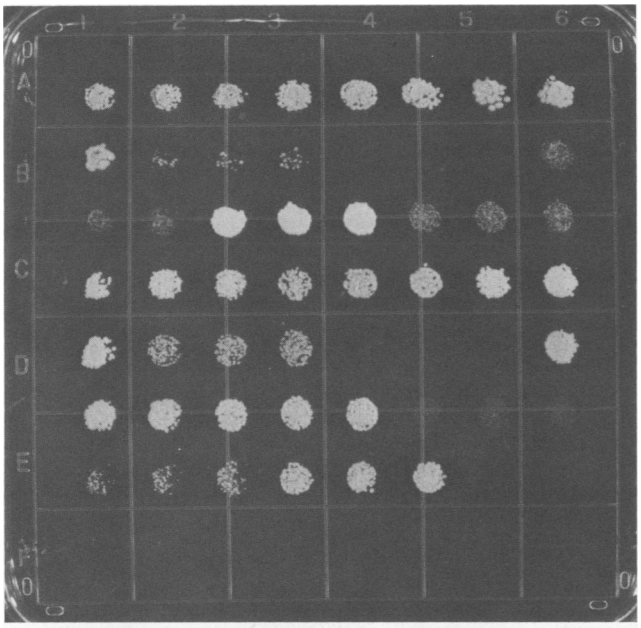

FIG. 1. Salt tolerance test plate 4 days after inoculation of the 18 reference strains in triplicate.

arranged in a rectangular configuration. Because the Clonemaster is rectangular rather than circular, agar plate tests were prepared by using square petri plates (100 by 100 by 15 mm; Baxter, McGaw Park, Ill.) instead of standard round petri plates as described in the original method. The first and last two columns on the Clonemaster do not fit into the square petri dishes and therefore were not used for inoculation. Utilization of the center eight columns allowed for the printing of 64 isolates, rather than 60 with the custom-built device of Odds and Abbott. This modification allowed for the processing of at least as many isolates as could be processed per plate in the original system.

For our study, media were prepared by the original method and formulation (7, 8) described by Odds and Abbott. The amount of test medium required for each square test plate was determined to be 30 ml. To dry agar surfaces, all plates except the proteinase production plates were incubated inverted for 1 h at 37°C. The proteinase production plates were not incubated, since drying caused cracking of the medium surface. Plates were either inoculated immediately or stored at 4°C for no longer than 2 weeks.

After each strain was adjusted to approximately 10^7 yeast cells per ml, the 1:10 dilution step was done in wells of a flat-bottom standard microtiter plate (Costar, Cambridge, Mass.). The prongs of the Clonemaster were sterilized by alcohol flaming and then immersed into the yeast cell inocula contained in the microtiter plate. Cells were transferred to test media by touching the prongs to the surface of the agar and applying even pressure and gentle agitation.

The Clonemaster delivered uniform and well-spaced clusters of colonies, as is evident in Fig. 1. Growth of each isolate is evaluated starting from the top left to right, row by row, and recorded as either positive for growth or negative for no growth. For each agar plate test, a positive and negative result has been clearly defined in the original paper of Odds and Abbott (7). As shown in Fig. 1, the Clonemaster delivered not only well-defined clusters of colonies but also uniform inocula. A triplicate set of one strain could be easily determined, and each strain had a characteristic growth pattern.

TABLE 1. Percent probability values for replication-to-replication variation in agar plate tests

Test	P^a (%)	
	Odds and Abbott[b] (n = 92)	This laboratory (n = 18)
pH 1.55 tolerance	0.0	0.0
pH 1.40 tolerance	5.0	2.4
Proteinase production	10.0	4.7
Flucytosine resistance	0.0	1.0
Urea assimilation	5.8	2.5
Sorbose assimilation	6.6	3.5
Salt tolerance	7.9	4.4
Citrate assimilation	8.7	7.8
Boric acid resistance	—[c]	5.3
Safranine resistance	7.9	11.3

[a] Calculated as described by Sneath and Johnson (10).
[b] Results from reference 7.
[c] Boric acid resistance was not included in the original system (7, 8).

To test the reproducibility of results with this modified technique, the 18 reference strains were repeatedly inoculated onto all media. Over a 6-month period, a total of 10 replications were made for most agar plate tests using four different batches of test media. Safranine resistance data were the only results based on only seven replicates with three different batches of this medium. Two sets of concentrates were used to prepare the four different batches of test media for all tests except for safranine resistance. All concentrates were frozen at −70°C after preparation. At the time of use, the concentrate was thawed to 25°C and added to nine parts sterilized 2.2% Bacto-Agar (Difco Laboratories, Detroit, Mich.) maintained at 56°C. Concentrate stored for as long as 6 months at −70°C gave consistent results between replications. The frozen concentrates also gave repeatable data when compared with freshly prepared concentrates.

To assess the reproducibility of this modified inoculating method, the percent probability between replicates (P) was calculated by the method of Sneath and Johnson (10). The calculated P values from this laboratory were compared with P values from the original paper by Odds and Abbott. In their work, P values were calculated from data of 92 strains based on six replicates with three different batches of test media (7). As shown in Table 1, the P values for the 18 strains tested in this laboratory were close to or under 5%, except for two of the most difficult agar plate tests, citrate assimilation and safranine resistance. Within a laboratory, a value of 5% or below for any given test is considered good reproducibility (10). A test with a P value of greater than 10 to 15% should be considered for rejection (10). In comparison to the study of Odds and Abbott, the P values overall from this laboratory using the modifications in suspending and inoculating yeast cells were lower, except for two tests, flucytosine and safranine resistance. In both laboratories, the flucytosine test is highly reproducible. The safranine resistance test showed low reproducibility in our hands, as it has for other laboratories (F. C. Odds, personal communication). These results, coupled with the difficulty in preparing the safranine media, suggest that substituting another test for this resistance test would improve the biotyping system.

In conclusion, the processes of suspending yeast cells and inoculating these cells onto test media were modified to

make this system more readily available and more economically feasible. The results of these modified methods indicate high consistency of yeast cell inocula and excellent reproducibility. These changes in methods improve the biotyping system and should allow for easier establishment of this system in other laboratories.

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