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Comparison of Various Techniques for Determining Viability of *Paracoccidioides brasiliensis* Yeast-Form Cells

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The viability of *Paracoccidioides brasiliensis* yeast-form cells was determined by colony-forming units, direct fluorescent staining, and production of germ tubes in slide culture. The first procedure was unreliable and time consuming; the latter two showed better correlation with hemacytometer total cell counts and required significantly less time.

The determination of the viability of yeast-form cells of the dimorphic fungus *Paracoccidioides brasiliensis* is usually performed by the colony-forming unit (CFU) procedure. This entails plating the inoculum onto an appropriate culture medium, which is incubated at room temperature (1, 4, 5, 7, 8). The development of mycelial colonies may require 3 to 4 weeks of incubation before counts can be performed (1, 6). The lack of correlation between hemacytometer counts and eventual number of colonies developed is a problem with this method (4), as might be anticipated since *P. brasiliensis* has mother cells with multiple buds. Thus, the determination of viability by CFU is not only a time-consuming procedure, but it also appears to be unreliable. This is unfortunate, because this information is necessary to accurately quantitate fungal populations that might be used for in vivo challenge, in vitro studies with antifungal agents, in vitro studies involving physical or chemical modification of the substrate, or in vitro studies with cellular or humoral components of host defenses. In searching for a faster and more dependable technique, we studied the fluorescent method described by Calich et al. (2) and also applied to *P. brasiliensis* yeast-form cells the microculture procedure that Sun and Huppert devised for genetic studies with *Coccidioides immitis* (9). This report presents the results of these studies.

Two human isolates of *P. brasiliensis*, LA and GI, were used. They were cultured in the yeast form at 36°C on slants of a synthetic medium modified from that described by McVeigh and Morton (MMcM medium) (7) and were regularly subcultured at 3-day intervals. This medium, either as the solid or the liquid formula, was used throughout the study.

A homogeneous suspension of young cells was obtained by transferring growth from one slant into a 125-ml Erlenmeyer flask containing 50 ml of liquid MMcM medium. This was incubated for 48 h at 36°C on a gyratory shaker (New Brunswick Scientific Co., New Brunswick, N.J.) at 200 rpm to disperse the clumps for more accurate cell counts by microscopy. After 48 h, the growth was subjected to mild sonication with a Branson Sonifier Cell Disrupter, model W-185, as described by Goihman-Yahr et al. (4). The dispersed cell suspension was centrifuged at 1,400 × *g* for 10 min, and the sedimented cells were resuspended in liquid MMcM medium by using brief (3 s) mechanical agitation with a Scientific Products, Inc., model S8220 mixer. Single cells constituted 75 to 93% of the resuspended cultures. The procedures for determining viability were initiated within 30 min of inoculum preparation.

A hemacytometer was used to count the total number of cells per milliliter. Viability testing was done by three methods. (i) CFU were determined as previously reported (1). Appropriate dilutions of the suspension were plated in duplicate on modified Sabouraud dextrose agar (3). (ii) Fluorescent microscopy was done by means of the fluorescein diacetate-ethidium bromide method described by Calich et al. (2). A Leitz Dialuz 20EB microscope equipped with filter-block I_2 for fluorescence was used. A total of 200 cells were counted to calculate the percentage of green-staining yeasts. These yeasts are considered viable. (iii) Assay by germination in slide culture was performed according to the procedure described by Sun and Huppert (9). By aseptic techniques, a block of solid MMcM medium (10 by 10 by 3 mm) was placed on a slide and inoculated with a 1:100 suspension of the

TABLE 1. Viability testing, by various methods, of *P. brasiliensis* yeast-form cells (isolate LA)

Expt no.	Hemacytometer counts/ml ($\times 10^6$)	Viable cells/ml ($\times 10^6$) determined by:		
		CFU	Germination in slide culture	Fluorescent microscopy
1	273	446.0	235	235
2	130	100.0	109	109
3	120	48.0	90	101
4	397	0.5	302	334
5	358	14.0	247	311
Mean values	255.6	121.7	196.6	218.0
Correlation index with hemacytometer counts		47.6	76.9	85.2

sonicated inoculum by use of a calibrated loop (0.001 ml). The inoculum was evenly dispersed by light pressure on the cover slip. The cover slip was sealed with lubricant and incubated in a humid chamber (a petri dish containing 2 ml of water) at room temperature (23 to 25°C). After 48 h, the slide was observed at 400× magnification, 200 cells were counted, and the percentage of yeasts giving rise to germ tubes was calculated. These yeasts are considered viable.

For both the fluorescent and microculture techniques, the percentage of cells that were viable was converted to the total number of viable cells by using the initial hemacytometer counts. A correlation index between hemacytometer count and viable cells was determined for the three methods by using the formula constructed by Goihman-Yahr et al. (4): (viable particles per milliliter)/(total cells in hemacytometer per milliliter) × 100.

Ten sets of experiments, five for each isolate, were performed. For isolate LA (Table 1) a wider variation in results was observed in the CFU experiments. In one experiment, there was almost twice the number of colonies as of cells counted in the hemacytometer, but in three others the number of colonies was only a fraction of the number of yeast-form cells in the initial hemacytometer count. The mean correlation index was 47.6. In general, results of the germination and fluorescein tests were in better agreement with the hemacytometer counts. The latter, as expected, were always larger, as they comprised both viable and nonviable cells. The correlation indices were 76.9 and 85.2 for the slide culture and fluorescent techniques, respectively.

CFU for isolate GI (Table 2) were much lower than for isolate LA, with an extremely low correlation index of 0.06. In the microculture and fluorescent tests, however, there was a closer relationship to the initial hemacytometer counts with this strain. Again the correlation indices were high: 69.9 for the slide culture and 91.1 for the fluorescent technique.

The results of this study indicate that, as earlier reported by Goihman-Yahr et al. (4), the CFU procedure is not a reliable measurement of the viability of *P. brasiliensis* yeast-form cells. Our studies, moreover, confirmed their findings

TABLE 2. Viability testing, by various methods, of *P. brasiliensis* yeast-form cells (isolate GI)

Expt no.	Hemacytometer counts/ml ($\times 10^6$)	Viable cells/ml determined by:		
		CFU ($\times 10^2$)	Germination in slide culture ($\times 10^6$)	Fluorescent microscopy ($\times 10^6$)
1	30.2	6.6	20.2	25.3
2	41.0	68.0	27.8	36.0
3	210.0	2000.0	149.1	199.5
4	34.0	210.0	22.7	29.5
5	39.0	100.0	27.8	32.4
Mean values	70.8	476.9	49.5	64.5
Correlation index with hemacytometer counts		0.06	69.9	91.1

despite our more exacting culture conditions, i.e., synthetic medium, controlled temperature, and inoculum.

The two microscopic techniques used in this study, on the other hand, were reproducible and apparently furnished the desired information on the viability of the yeast population in an inoculum. Additionally, this was accomplished in significantly less time than is required for the CFU, approximately 0.5 h for the fluorescent stain and 48 h for the germination of yeasts in slide culture.

When one needs rapid results, the fluorescent technique has certain advantages. However, appropriate equipment must be available, and a well-dispersed inoculum is essential. It is impossible to accurately count within aggregates of cells in undispersed preparations. The slide culture procedure is free of this complication. When germinating and nongerminating cells (or groups of cells) are in the same optical plane for microscopic observation, germination can be distinguished clearly. This technique may also have special advantages for studying interactions between phagocytic and fungal cells or the effects of chemical factors, physical factors, or both on the development of yeasts.

In summary, the fluorescein diacetate-ethidium bromide staining method and the production of germ tubes in slide culture are dependable procedures which are easy to perform to determine the number and viability of *P. brasiliensis* yeast-form cells. These methods may also be useful for the study of other dimorphic fungi.

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