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A NEW FLUORESCENT VIABILITY TEST FOR FUNGI CELLS

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Abstract

The fluorescein diacetate-ethidium bromide (FDA-EB) fluorescence method, primarily used to determine viability of mammalian cells, was applied to several fungi species. Living fungi cells produced fluorochromasia, i.e., an intracellular accumulation of fluorescein which could be easily visualized as a green color under the U.V. microscope. Dead cells showed a red bright color due to ethidium bromide penetration. The FDA-EB test can be successfuly employed to assay yeast and yeast like cells viability since a good correlation was observed between this assay and the colony count technique. The main advantages of FDA-EB test are its speed, high sensitivity and simplicity.

Introduction

The different type on living and dead animal cells in cytotoxic tests usually employs dye exclusion test such as eosin 'Y' and trypan blue (5). On the other hand, the colony count technique of Miles and Misra (4) is a very useful test for assaying the viability of microorganism cell suspensions.

Celada and Rotman (1) described a cytotoxic test based on fluorochromasia (6), the property of living mammalian cells to accumulate fluorescein intracellularly as a result of enzymatic hydrolysis of a fluorogenic substrate (fluorescein diacetate, FDA). Another fluorescent compound, ethidium bromide (EB), was used by Edidin (2). While EB penetrates intact cells slowly, it rapidly enters damaged cells giving a bright red fluorescence. In 1971, Takasugi (7) improved the cytotoxic test by combining both compounds, FDA and EB in order to obtain a strong contrast between the living cells (green fluorescence) and the dead ones (red fluorescence).

The present investigation was undertaken to determine the usefulness and sensitivity of this fluorochromatic assay to assess the viability of fungi cells in relation to the usual colony count method.

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Material and methods

Organisms and cultural conditions

The following species of fungi were used: *Candida albicans* (ICB 7, 12, 61), *Candida guilliermondii* (ICB 9), *Candida parapsilosis* (ICB 15), *Saccharomyces cerevisiae* (ICB 39), *Hansenula anomala* var. anomala (ICB 29), *Sporothrix schenckii* (ICB 16), *Cryptococcus neoformans* (ICB 63), *Paracoccidioides brasiliensis* (ICB 18), *Penicillium* sp (ICB 89), *Rhodotorula rubra* (ICB 36) and *Aspergillus niger* (ICB 35). All species with exception of *P. brasiliensis* were grown in Sabouraud dextrose agar at room temperature. The yeast were harvested after 48 hr cultivation and molds were obtained from one week old cultures. The yeast like form of *P. brasiliensis* was cultivated one week on Fava Netto's medium (3) at 37 °C.

Microorganisms were harvested, washed three times in phosphate buffered saline, pH 7.4 (PBS), by centrifugation at 200 g for 10 min and counted in a hemocytometer. Aliquots from saline suspensions of all fungi assayed were sterilized by autoclavation in order to get a reference standard of dead cells.

Reagents

The stock solution of fluorescein diacetate (Calbiochem, Los Angeles) was prepared in acetone at a concentration of 5.0 mg/ml and kept at –20 °C. Ethidium bromide (2, 7

diamino-10-ethyl-9-phenylphenanthridinium bromide; Sigma, London) solution was prepared by dissolving the compound in PBS at a concentration of 50.0 μg/ml. Just before use, the FDA solution was diluted to 1 : 2, 500 in PBS and mixed to equal volume of EB solution.

Viability test

Equal volumes of the FDA-EB solution and the fungi suspensions were mixed and incubated. Several incubation periods and different temperatures (37 °C, 25 °C and 4 °C) were investigated. One drop of suspension was placed over a slide, covered with a cover slip and examined under a Zeiss microscope equiped with ultraviolet light, BG-12 exciter and Zeiss 50 barrier filters. One hundred cells were counted in each slide and the percentage of dead (red) cells was evaluated. For comparison, the viability of some yeast cells suspensions were also assayed by the Miles and Misra test. All tests were performed in duplicate.

Results

Initial results showed that the most intense fluorochromatic reaction was obtained with yeast like cells as *P. brasiliensis*, *Candida spp*, *S. cerevisiae* and *Rhodotorula rubra*.

C. neoformans, *H. anomala* and *S. schenckii* developed a slow green fluorescent reaction with lower intensity than *P. brasiliensis* cells. The latter showed a good fluorescence pattern within 15 min incubation with the stain reagents whereas the other fungi required 60 min or more.

This method was not readily applicable for the examination of the viability of mold cultures, due to the difficulty in obtaining good suspensions of unclumped cells. It was verified that with a one week old *Aspergillus niger* culture, all of the spores and 50% of mycelial cells presented the green fluorescence staining.

Relating to the temperature, the incubation at 37 °C gave the best results and, at 4 °C, the fluorescence development was very poor.

The results expressed in Table 1 show some variations between FDA-EB assay and the colony count method except a *C. albicans* sample (ICB 12) that presented identical results.

In control reactions in which heat killed cells were used, only EB red fluorescence was seen.

Table 1 – A comparison of viability determination using colony count technique versus FDA-EB staining.

Yeast	Viability	
	FDA-EB*	Colony Count
1. *C. albicans* (ICB 7)	6.7×10^7/ml	8.5×10^7/ml
2. *C. albicans* (ICB 61)	4.0×10^7/ml	2.3×10^7/ml
3. *C. albicans* (ICB 12)	1.2×10^7/ml	1.2×10^7/ml
4. *C. guilliermondii* (ICB 9)	12.7×10^7/ml	17.7×10^7/ml
5. *S. cerevisiae* (ICB 39)	3.3×10^7/ml	2.2×10^7/ml

*Incubations were carried out for 1 hr at room temperature.

Discussion

The present investigation showed that the FDA-EB test can be successfully employed to assay the yeast and yeast like cells viability. The test presented had several advantages due to its speed, high sensitivity and simplicity as compared to the colony counting technique.

The best fluorochromatic reactions were obtained with the yeast phase of *P. brasiliensis* that required short period of incubation (15 min) with the fluorescent reagents. It was also verified that aging the *P. brasiliensis* cells in growth medium or PBS resulted in an increasing inability to accumulate fluorescein. These results correlated with a progressive loss of *P. brasiliensis* viability. These are the most remarkable aspects of practical FDA-EB technique observed. Undoubtedly, this test overcome the difficulties found in classical determination of those microorganism/cell viability. The use of two contrasting fluorescent reagents provides remarkably clear results not obtained in other viability assays.

Good fluorochromatic reactions were also obtained with *C. albicans* and *S. cerevisiae* with 60 min of incubation with EB-FDA at 37 °C.

The variations in the results of the colony counting assay and the fluorescence assay (Table 1) may be due to the fact that these tests measure different parameters. While the colony counting test measures the growth rates of the cells, the fluorescence assay reflects the internal enzyme content and the permeability of the cells. It seems that the FDA-EB results are the most realistic ones because they are the expression of what is happening to the original suspensions at a particular time.

Different fungi species showed several degrees of green fluorescence when assayed in the same experimental conditions. This fact could be explained by their different content in cytoplasmatic sterases which are responsible for

the FDA enzymatic hydrolysis, giving green fluorescence in the viable cells.

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