Comparison of Four Methods for Determining Nitrate Utilization by Cryptococci

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This study evaluated the following methods for determining nitrate utilization: Wickerham broth, a special nitrate broth, Delft plate, and nitrate strip. With 236 isolates of cryptococci as test organisms, the special nitrate broth method gave 99% correct results and the Wickerham broth method gave 98%. The nitrate strip and Delft plate methods gave correct results in 94 and 86% of tests, respectively. The special nitrate broth method is judged superior because it provides accurate results within 48 h, compared to 14 days with the Wickerham broth method.

Most of the methods currently available for determining nitrate utilization by cryptococci have disadvantages because the test is difficult to read or an extended incubation period is required. In this study we compared the Wickerham broth test, a modified Delft plate test, a special nitrate broth, and a commercially available nitrate strip method in regard to the following aspects: (i) reliability of results, (ii) time required for performance of the test, and (iii) simplicity of interpretation.

MATERIALS AND METHODS

Cultures. The distribution of the 236 identified isolates of cryptococci that were used is as follows: Cryptococcus neoformans 190, C. laurentii 14, C. luteolus 5, C. albidos var. albidus 9, C. albidos var. diffluens 12, C. terreus 5, and Cryptococcus species 1. These isolates were obtained from the Mayo Clinic culture collection, from the Center for Disease Control, and from the culture collection of the late M. L. Littman. All organisms were kept in stock culture on modified Sabouraud's dextrose agar throughout the study. Before use, each organism was subcultured onto a fresh slant of modified Sabouraud's dextrose agar and incubated for 24 to 48 h at 30 C. The cultures used for inocula were transferred directly to nitrate utilization media without prior starvation. Throughout the entire study, a culture of C. albidos var. diffluens (CR-23) was used as a positive nitrate assimilation control organism.

Nitrate assimilation methods. The Wickerham broth method (3) was performed using yeast carbon base (Difco) supplemented with 0.078% potassium nitrate. After incubation for 1 week at 30 C, cultures showing nitrate assimilation were transferred to a fresh tube of medium by using a 0.01-ml calibrated loop. These cultures were incubated for 7 additional days before they were considered to assimilate nitrate.

The Delft plate method (2) used yeast carbon base (Difco) containing 2% Noble agar. Plates which contained 9 ml of Noble agar and 2 ml of 10× yeast carbon base were flooded with a suspension of yeast prepared in sterile physiological saline. The excess suspension was removed with a sterile capillary pipet and the surface of the plate was allowed to dry. A sterile 12.7-cm blank disk (Difco) was dipped in 1% peptone (Difco) solution and placed on the periphery of the plate for use as a positive control. Another blank disk was dipped in 1% potassium nitrate and placed in the center of the plate. After 24 h of incubation at 30 C, the plates were observed for growth around the disks. Some of the saprobic cryptococci required 24 h of additional incubation.

The Pathotec (General Diagnostics Division, Warner-Lambert Co., Morris Plains, N.J.) nitrate strip method was performed by adding 0.5 ml of a heavy suspension of the organism in sterile physiological saline to a sterile tube, with a reagent strip placed in the tube so that the lower reagent band was immersed in the suspension. The tube was incubated for 5 h at 30 C and then tipped to wet the upper reagent band containing the nitrate detection system. A pink to red color change was interpreted as a positive result. Zinc dust was added to the remaining suspension to check for false-negative reactions due to complete nitrate reduction (4).

A special nitrate broth medium was prepared as follows: indole nitrate medium (BBL) (12.5 g), soluble starch (5.0 g), sodium chloride (2.5 g), gelatin (10.0 g), dibasic potassium phosphate (0.5 g), and distilled water (500.0 ml). Two milliliters of this broth was dispensed per tube, and the tubes were autoclaved for 15 min at 121 C. The tubes were heavily inoculated and incubated at 30 C for 48 h. Six drops each of the sulfanilic acid and o-naphthylamine reagents described by Edwards and Ewing (1) were added to each tube; each tube was shaken and observed for the presence of a red color that indicated nitrate reduction. A small amount of zinc dust was added to each negative test for confirmation.

RESULTS

Table 1 lists the results by species for each method tested. The cumulative results were
that the special nitrate broth gave 99% correct results, followed by 98% given by the Wickerham broth method and 94 and 86% given by the nitrate strip and Delft plate methods, respectively. The special nitrate broth method detected nitrate utilization with only two of the five C. terreus isolates; however, it yielded correct results in all other instances. The Delft plate method gave false-positive results with 27 of the 190 C. neoformans isolates tested.

**DISCUSSION**

*Cryptococcus neoformans* is the most important member of the genus to be identified in a clinical laboratory. One important criterion for the definitive identification is the inability of the organism to utilize an inorganic nitrate source. Our objective was to evaluate current methods for detecting nitrate utilization and to determine which would provide most accurate results and could be easily interpreted in a short period.

The special nitrate broth and Wickerham broth methods provided the most accurate results, and agreement between the two was high. No false-positive results for nitrate assimilation by *C. neoformans* were provided by either method. The time required to achieve a meaningful result was 48 h for the special nitrate broth in contrast to 14 days for the Wickerham broth method. The latter uses yeast carbon base which supports the growth of some cryptocoeci and permits a background growth that makes interpretation of the test difficult. Those organisms that utilized nitrate in the special nitrate broth method always produced a color change within 5 min.

The time required for completion of the nitrate strip method was approximately 5 h, but this test gave false-positive results with 7 of 190 isolates of *C. neoformans*. In general, the strips gave distinct color changes when positive; however, some difficulty in interpretation was noted due to the production, during incubation, of a slight pink color that was attributed to exposure to light and moisture.

The Delft plate method proved to be the least satisfactory. Twenty-seven of 190 isolates of *C. neoformans* produced false-positive nitrate utilization reactions on this medium. It was very difficult to distinguish differences in the intensity of growths around the potassium nitrate test disk and the positive control disk. The yeast carbon base allowed the organisms to produce just enough background growth to be considered positive.

We recommend that the special nitrate broth method be used as a means of detecting nitrate utilization by cryptocoeci. We have now extended the incubation time to 72 h because some slower-growing isolates need this additional time. The method is simple, accurate, and rapid, and it is practical for use in a clinical microbiology laboratory.

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**LITERATURE CITED**