Medium Containing Trypan Blue and Antibiotics for the Detection of Cryptococcus neoformans in Clinical Samples

RICHARD M. VICKERS, JAMES J. McELLIGOTT, JR., JOHN D. RIHS, AND BOSKO POSTIC

The Laboratory and Medical Services of the Veterans Administration Hospital, Pittsburgh, Pennsylvania 15240, and the Departments of Pathology (Microbiology Section) and Medicine of the University of Pittsburgh, School of Medicine, Pittsburgh, Pennsylvania 15261

Received for publication 31 May 1973

A medium containing trypan blue, gentamicin, and chloramphenicol is introduced for the detection of Cryptococcus neoformans and Cryptococcus species from clinical samples. Ten recently isolated strains of Cryptococcus species as well as several clinical isolates of C. neoformans incorporated trypan blue and produced dark blue colonies on this mycological medium, whereas other common yeasts were light blue. The laboratory diagnosis of two cases of cryptococcosis was accomplished by the isolation of C. neoformans on the antibiotic-dye-containing medium. Compared to conventional media supporting large numbers of Pseudomonas aeruginosa and other gram-negative bacilli, the new medium was selective for yeasts. In one instance, the colonization of the respiratory tract by C. neoformans which led to fungemia was traced by the use of the antibiotic-dye medium. The antibiotic mixture, utilized herein, was more effective in suppressing bacteria contained in samples from patients than a medium containing cycloheximide and chloramphenicol.

The purpose of this investigation was to develop a mycological medium for the primary isolation of Cryptococcus neoformans. To accomplish this, the medium had to be optimal for the growth of the yeast, the colonies had to be distinguishable by a preferential dye uptake, and competing bacteria were to be suppressed.

Media containing dyes are not commonly used for the primary isolation of fungi. A trypan blue-containing chlamydospore agar is used to identify the morphology of Candida species, rather than for their primary recovery (7). The use of this medium for the identification of all yeasts isolated in this diagnostic laboratory revealed that C. neoformans and other Cryptococcus species produced dark blue growth whereas other yeasts usually did not (R. M. Vickers, unpublished data). Since chlamydospore agar is suboptimal as a growth medium, the dye was added to commercial mycological media, to examine whether the uptake of trypan blue by species of Cryptococcus was consistent.

To inhibit bacteria and thus facilitate the isolation of pathogenic fungi including C. neoformans, gentamicin and chloramphenicol were added to brain heart infusion agar, as recommended by Dolan (1), and to other media as well. Reported herein is the selection and use of a medium containing trypan blue as well as gentamicin and chloramphenicol for the speedy detection and tentative identification of C. neoformans in samples from patients in the Veterans Administration Hospital, Pittsburgh, Pa.

MATERIALS AND METHODS

Antibiotics. Gentamicin (Schering Corp.), in vials of 500 mg as sulfate powder (550 μg/mg), and chloramphenicol sodium succinate, in 1-g vials (Park-Davis), were dissolved at 10,000 μg/ml according to the manufacturer's instructions and stored at −8 C before incorporation into media.

Media. BBL and Difco Sabouraud dextrose agar (SAB), BBL mycophil agar (BBL Myc), Difco mycological agar (Difco Myc), and BBL and Difco brain heart infusion agar (BHI) were used. Trypan blue no. NA 0508 (Allied Chemical) was added unfiltered at 0.1 g of dye per liter of medium. Gentamicin and chloramphenicol were added to media at 10 μg and 20 μg per ml, respectively, and are referred to as 10/20 media. No antibiotics were added to SAB, and 2.5 g of disodium phosphate per liter was added to the BBL Myc and Difco Myc agars only (see Results).

Organisms. Ten strains of Cryptococcus species were selected for study: C. neoformans (5 strains), C. diffluens (2 strains), C. laurentii (2 strains), and C. albicans (1 strain). Eight were isolated in this laboratory, whereas one strain each of C. neoformans and C. albicans were kindly supplied by H. Layman of Montefiore Hospital, Pittsburgh, Pa. Yeasts were identified according to current criteria (9). A strain of
**Candida albicans** and an overnight Trypticase soy broth (BBL) culture of *Pseudomonas aeruginosa* were also used. The MIC of gentamicin for the *P. aeruginosa* indicator strain was 5 μg/ml on BBL Trypticase soy agar. To monitor the gentamicin activity of 10/20 media, the growth of the indicator strain was compared to that on the corresponding medium without antibiotics. Five colonies or more on 10/20 medium was interpreted as lack of antibacterial effect.

**Inocula.** Each strain of Cryptococcus sp. and *C. albicans* was suspended in 1 ml of sterile saline aiming at the same turbidity. The suspensions contained approximately 25 cells per microscope field viewed through the ×45 objective. Two types of inocula were used: (i) each Cryptococcus sp. alone, (ii) a mixture of each *Cryptococcus* sp. and *C. albicans* suspension in equal amounts. Inocula were delivered onto plates by a 2-mm diameter loop and streaked by use of a four-quadrant pattern. The plates were incubated at 22 to 25 C, observed daily with a final readout at 7 days. Color and colony sizes were recorded for comparison.

**Trials with clinical samples.** This laboratory uses, for fungi, Difco (SAB) agar (no antibiotics) and BBL mycoser agar (contains cycloheximide and chloramphenicol) as slants in 1-ounce (approximately 30 ml) prescription bottles. Each 10/20 medium was also prepared in bottles and inoculated in parallel with those routinely used. One experimental medium was used at a time for a period of 2 weeks to determine its suitability for continued study. The cultures were incubated for a total of 30 days at 22 to 25 C to evaluate color changes of yeast colonies. Concurrently, plates containing commercial media with the addition of trypan blue (with and without antibiotics) were inoculated and incubated (22 to 25 C) for periods of 7 days or shorter.

**RESULTS**

**Growth of Cryptococcus sp. on media with dye and antibiotics.** Table 1 lists the average diameters (in millimeters) of 5 isolated colonies of *Cryptococcus* sp. on medium without antibiotics, but containing trypan blue. The best medium for growth was SAB, which had the largest average colony size. The smallest colonies were on BHI, with medium-sized colonies on BBL Myc and Difco Myc. *Cryptococcus* sp. colonies grew slower than *C. albicans*, but the disparity in size diminished after 7 days of incubation. The least amount of capsular material, as estimated by an India ink preparation, was produced on BHI agar. The addition of gentamicin and chloramphenicol did not affect the colony sizes.

In respect to the uptake of trypan blue, the following was observed. (i) *Cryptococcus* sp. grew as dark blue colonies on all media listed in Table 1. They appeared after 24 h, and became darkest at 48 h and later. Growing colonies remained uniformly blue, indicating that the incorporation of the dye was continuous (for exception, see below). (ii) When grown in pure culture, cryptococcal colonies lost some color after 5 days on SAB only, with the base remaining as a dark blue circle. (iii) *Cryptococcus* sp. colonies were shiny and wet at onset but became opaque and pasty after 4 to 5 days, as is typical for a yeast. (iv) Colonies of *C. albicans* were light blue, dull, and opaque. (v) When mixed with *C. albicans*, the colonies of *Cryptococcus* sp. were darker on each medium than when grown in pure culture. (vi) When trypan blue was omitted from media, the colonies of *Cryptococcus* sp. were identical in size to those grown on dye-containing plates.

Figure 1 is an enlarged photograph of *C. neoformans* and *C. albicans* colonies on SAB after 7 days. The differential uptake of trypan blue by *C. neoformans* is evident. Its dark colonies contrast to the pale *C. albicans*.

**Selection of media maintaining the activity of gentamicin.** The indicator strain of *P. aeruginosa* was inoculated onto media to which antibiotics (10/20) had been added. The indicator strain was not suppressed on SAB plates. This could be due to the low pH of the medium (5.5), which decreases the antibacterial effect of gentamicin (3). Therefore, SAB was eliminated as a selective medium for the isolation of *C. neoformans* from clinical samples. The other media were modified as follows. It was necessary to add 2.5 g of disodium phosphate per liter to BBL Myc and Difco Myc to raise the pH of the media from 6.9 to 7.2 for effective gentamicin activity. With added gentamicin and chloramphenicol, BBL Myc and Difco Myc were equally capable of supporting the growth of *Cryptococcus* sp., as well as suppressing bacterial growth. Either medium is referred to, hereafter, as Myc 10/20.

### Table 1. Comparative growth of Cryptococcus sp. in mycological media containing trypan blue

| Cryptococcus sp. | Strains (no.) | Mean colony diameter (mm)* | SAB | BBL-Myc | DIFCO-Myc | BHI |
|-----------------|--------------|---------------------------|-----|---------|-----------|-----|
| *C. neoformans* | 4            |                          | 6   | 5       | 5         | 3   |
| *C. neoformans* | 1*           |                          | 3   | 2       | 2         | 1   |
| *C. diffluens*  | 2            |                          | 6   | 5       | 5         | 3   |
| *C. laurentii*  | 2            |                          | 6   | 5       | 5         | 3   |
| *C. albicus*    | 1            |                          | 6   | 5       | 5         | 3   |

* After 7 days at 22 to 25 C; the media are described in Materials and Methods.

*This strain is a consistent “slow grower.”*
Most importantly, *C. neoformans* was isolated repeatedly from two infected patients during the trial of Myc 10/20. To illustrate the diagnostic adequacy of this medium, the cases are summarized below.

In case 1, during and preceding the 48-h-long terminal febrile episode affecting a 49-year-old leukemic, there were no clinical signs of cryptococcosis, such as pneumonia, pyelonephritis, or meningitis. Blood cultures drawn antemortem were positive for *C. neoformans*. Specimens from the lungs, brain, heart valve, and blood obtained at autopsy yielded *C. neoformans* on conventional media as well as on Myc 10/20. The stool was also cultured; gram-negative bacilli overgrew completely media without antibiotics but five dark blue colonies of *C. neoformans* were isolated on Myc 10/20, indicating its effectiveness as a selective medium. Significantly, the *C. neoformans* isolated on Myc 10/20 from each of the above samples incorporated trypan blue, whereas it was not isolated on the cycloheximide-containing medium as expected (4).

In case 2, another instance of successful and repeated isolation of *C. neoformans* was from a 56-year-old patient whose stormy postoperative course (postresection and -grafting of the thoracic aorta) included episodes of acute tubular necrosis, uremia, and septicemia due to *Serratia marcescens*. This was treated with gentamicin and ampicillin. Although the bacterium could no longer be isolated from the blood, respiratory difficulties and signs of pneumonia appeared. The sputum (or tracheal aspirate) and blood were repeatedly cultured during the last 3 weeks of life. Table 2 lists results of cultures relating to *C. neoformans*. The first sputum listed (3/4/73) yielded on Myc 10/20 10 colonies of *C. neoformans* (dark blue colonies), 10 colonies of *C. albicans* (light blue colonies), and approximately 100 colonies each of gentamicin-resistant *P. maltophilia* and *Herellea (Bacterium anitratum)*. Subsequent sputum cultures were readily recognized as positive for *C. neoformans* on Myc 10/20, whereas conventional media seeded with the same samples were overgrown with gram-negative bacilli including *P. aeruginosa* or *C. albicans*. On 3/12/73, the patient became markedly febrile (40.6°C) and blood cultures yielded *C. neoformans* consistently (Table 2). Intravenous amphotericin B was administered in increasing daily doses of 5, 15, and 25 mg, after which the patient expired. At autopsy, unlike the preceding case, *C. neoformans* was isolated in scant numbers from the lung, probably due to the

---

**Fig. 1. Colonies of Cryptococcus neoformans (dark) and Candida albicans (light) on Sabouraud dextrose agar containing trypan blue after 7 days at 22 to 25°C.**
fungicidal effect of amphotericin B. The predominating organism cultured from the lungs, showing extensive bilateral pneumonia with two abscesses (3 to 5 cm in diameter), was *P. maltophilia* resistant to gentamicin. With a microscope, abundant yeast forms characteristic of *C. neoformans* were seen in the abscess wall and cavity, leaving no doubt that it was the cause of necrotizing pneumonia.

**DISCUSSION**

The medium, Myc 10/20, introduced here for the isolation of *C. neoformans* contains trypan blue, gentamicin, and chloramphenicol. It allows the detection of *C. neoformans* and other *Cryptococcus* species as soon as colonies appear without affecting the viability of the yeast. The incorporated antibiotics inhibit most bacteria in clinical samples. Hence, *C. neoformans* grew on Myc 10/20 uninhibited. The uptake of trypan blue by *C. neoformans* and *Cryptococcus* sp. readily differentiates them from most yeasts. In respect to the dye uptake, the 10 strains tested here and those isolated from the clinical trial conducted in the course of this research appear representative of most *Cryptococcus* species. The only yeast and yeast-like organisms yielding a growth as dark as a *Cryptococcus* sp. are *Trichosporon cutaneum* and *Rhodotorula* species (R. M. Vickers, unpublished data). These are differentiated by colony morphology, color (on medium without dye), assimilation, and fermentation tests (9). The reason(s) for the dye uptake by the above organisms was not investigated and remains unknown.

The use of trypan blue as an aid in the recognition of *Cryptococcus* sp. isolates has not been attempted before. Attempts to single out *Cryptococcus* sp. by color on a medium are few. Shields and Ajello (8) recommended a medium containing a pigment derived from *Guizotia abyssinica* seeds, creatinine, and chloramphenicol for the isolation of *C. neoformans*. It was the only yeast which assimilated creatinine and consistently produced brown colonies due to the uptake of pigment. No mention was made whether gentamicin could be used for the suppression of gram-negative bacilli, particularly *P. aeruginosa* in clinical samples. In contrast, the Myc 10/20 medium was shown to be applicable for the primary isolation of *Cryptococcus* sp.

A disadvantage of the Myc 10/20 medium is the relatively high concentration of Mg$^{2+}$ and Ca$^{2+}$, by spectrophotometer 4.3 and 11.1 mg/100 ml, respectively, which reduces the gentamicin activity against *P. aeruginosa* (5). Dolan recommended the incorporation of 4 µg of gentamicin per ml into BHI agar for the isolation of fungi (1). To exceed the MIC of 90% or more of clinical isolates of gram-negative bacilli encountered in this laboratory, the gentamicin was increased to 10 µg/ml in the 10/20 medium. This appears optimal since Dolan demonstrated that *C. neoformans* was significantly suppressed at 16 µg of gentamicin per ml (1).

The isolation of *C. neoformans* from the sputum of one of the cases presented here (see Results) came from our current study aimed at determining the prevalence of *C. neoformans* in spuota and its relationship to clinical cryptococcosis. Although noninvasive pulmonary cryptococci were previously observed by Finegold and co-workers (2), one of the patients studied herein developed fungemia 1 week after scant *C. neoformans* was detected in respiratory secretions. It is not known whether the recovery of *C. neoformans* from the sputum of patients without pulmonary lesions could predict systemic cryptococcosis. If invasion occurred in a significant proportion of colonized patients, early treatment with an antifungal agent may be indicated, particularly in cases with impaired cell immunity that are at special risk (6). The medium containing dye and antibiotic provides a sensitive tool to monitor colonization of the respiratory tree with *C. neoformans* and trace the infection from this portal of entry to fungemia and meningitis. Although saprophytic

**TABLE 2. Repeated recovery of *C. neoformans* from the sputum and blood of a patient**

| Date (1973) | Sputum* colonies/ Myc-10/20 plate | Blood* | Sputum Colonies/ ml |
|------------|----------------------------------|--------|-------------------|
| Mar 4      | 10                               | ND     | ND                |
| Mar 5      | ND*                              | ND     | ND                |
| Mar 5      | ND                               | ND     | ND                |
| Mar 12     | ND                               | +      | 2                 |
| Mar 12     | ND                               | +      | 2                 |
| Mar 13     | 50                               | +      | 0                 |
| Mar 16     | 100                              | ND     | ND                |
| Mar 16     | 100                              | ND     | ND                |
| Mar 18     | ND                               | +      | 20                |
| Mar 19     | 100                              | ND     | ND                |
| Mar 20     | 100                              | ND     | ND                |

* Sputum was cultured by swab on both conventional and Myc 10/20 plates; the former yielded only gram-negative bacterial growth.
* Four milliliters was squirted into a bottle containing 50 ml of Trypticase soy broth (BBL) and 1 ml was mixed with 10 ml of agar at 50 C and plated.
* ND, Not done.
* Refers to growth (+) or no growth (−).
Cryptococcus sp. incorporate trypan blue, the presence of dark blue colonies suggests the presence of the opportunistic pathogen, C. neoformans, in clinical samples, and is therefore of value at isolation.

ACKNOWLEDGMENT

We appreciate the able assistance of the technical staff of the Microbiology Section, particularly Roland Robinson, the Renal Research Unit, and the photography by Medical Illustration at the Veterans Administration Hospital, Pittsburgh, Pa. We thank Monto Ho, H. Richard Hellstrom, and George T. Pazin for their help in the preparation of the manuscript.

LITERATURE CITED

1. Dolan, C. T. 1971. Optimal combination and concentration of antibiotics in media for isolation of pathogenic fungi and Nocardia asteroides. Appl. Microbiol. 21:195-197.
2. Finegold, S. M., D. Will, and J. F. Murray. 1969. Aspergillosis: review and report of twelve cases. Amer. J. Med. 27:463-482.
3. Finland, M. 1969. Gentamicin: antibacterial activity, clinical pharmacology and clinical applications. Med. Times Port. Wash. N. Y. 97:161-174.
4. Georg, L. K., L. Ajello, and C. Papageorge. 1954. Use of cycloheximide in the selective isolation of fungi pathogenic to man. J. Lab. Clin. Med. 44:422-428.
5. Gilbert, D. N., E. Kutscher, P. Ireland, J. A. Barnett, and J. P. Sanford. 1971. Effect of the concentrations of magnesium and calcium on the in-vitro susceptibility of Pseudomonas aeruginosa to gentamicin. J. Infect. Dis. 124:S37-S44.
6. Louria, D. B. 1971. Superinfection due to fungi. p 147-152. In Proc. Int. Conf. Nosocomial Infections, Amer. Hosp. Ass. Chicago, Ill.
7. Nickerson, W. J., and Z. T. Mankowski. 1953. Polysaccharide medium of known composition favoring chlamydospore formation in Candida albicans. J. Infect. Dis. 92:20-25.
8. Shields, A. B., and L. Ajello. 1966. Medium for selective isolation of Cryptococcus neoformans. Science 151:208-209.
9. Silva-Hunter, M. 1970. Yeasts, p. 352-363. In J. E. Blair, E. H. Lennette, and J. P. Truant (ed.), Manual of clinical microbiology. American Society for Microbiology, Bethesda, Maryland.