Tetrazolium medium as an aid in the routine diagnosis of *Candida*¹

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SYNOPSIS The tetrazolium medium described is easily prepared and serves as a rapid, relatively accurate and simple means of differentiating *Candida* species, particularly *C. albicans*, from other yeasts. Culture on this medium does not replace other techniques used for yeast identification. No one test alone will suffice and final identification must rest on an overall pattern of characteristics. In the screening of 1,822 yeast isolates in this laboratory, culture on this medium has proved a valuable adjunct to other recognized techniques, and is now in regular use.

The rapid identification of *Candida* species is a common problem in routine laboratories. Not every yeast-like fungus seen in direct tissue smears or in culture is a *Candida*. Nor can it be assumed that in the isolation of *Candida* from man, only *C. albicans* is pathogenic and need be identified, since other species of *Candida* (*C. tropicalis*, *C. pseudotropicalis*, and *C. stellatoidea*) are known from experimental evidence to be pathogenic and have been recovered from disseminated infection in man (Hurley and Winner, 1964).

No single test is sufficient for full identification of *Candida* species (Drouhet). Yeasts not identified as *Candida albicans* by the germ-tube test (Reynolds and Braude, 1956) should not be discarded. If time allows, deep streaks in cornmeal agar will serve to identify *C. albicans* and other *Candida* species by their mycelial development and morphology, and sugar fermentations may be undertaken.

Pagano, Levin, and Trejo (1958) found that tetrazolium salts could be used as biological indicators to differentiate *Candida* species, depending on their ability to reduce them to insoluble, coloured formazans, deposited intracellularly. Although 'Pagano-Levin' medium, a nutrient agar incorporating a monotetrazolium salt, is relatively widely used for *Candida* studies in America, it is little used in Britain. Our assessment of this medium as an aid in laboratory diagnosis was made during the course of routine identification of yeasts isolated

from patients attending at the Charing Cross Group of Hospitals.

METHODS AND MATERIALS

The yeasts received in our laboratory for further investigation were those isolated in the routine bacteriology laboratories, usually on peptone-dextrose agar (without antibiotics) and sometimes blood agar, and usually subjected to a preliminary screening test for germ-tubes (2-3 hr in horse serum at 37°C) to detect probable *Candida albicans* strains.

On receipt of the yeast culture plates, the following procedures were adopted: macroscopically different colonial forms of yeasts were picked off and (a) streaked into cornmeal agar plus 0.02% Tween 80 (deep streaks cut at an angle, without cover-slips or agar overlays) and incubated at 25°C; (b) plated out on to tetrazolium medium and incubated at 37°C.

To prepare the medium, dissolve 4% dextrose, 1% peptone (mycological), 0.1% beef extract (Lab. Lemco) in water, add agar (1.5 to 2%) and autoclave (10 lb for 10 min). This nutrient medium may be held as stock and melted as required. To the melted, partially cooled agar, add 500 µg/ml of neomycin and 200 µg/ml of 2, 3, 5-triphenyltetrazolium chloride (TTC). The stock solution is made up of 100 mg/ml in distilled water, Seitz filtered and stored at +4°C. Final pH 5.5 to 6.0.

On average, six to eight colonies were tested on each type of agar plate. Cornmeal plates were examined at 24-hour intervals for the presence of chlamydospores. Tetrazolium plates were examined for growth and colour changes after 24 hours (Fig. 1).

It was usually possible to differentiate as non-*Candida* species, those yeasts which failed to produce mycelium on cornmeal and gave poor growth on tetrazolium medium, although some produced coloured colonies.

Where confirmation of *Candida* species other than

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FIG. 1. Growth of *Candida* species on tetrazolium medium after 24 hours at 37°C. *Candida albicans*, 1, 3, 5; *C. tropicalis*, 2, 7; *C. krusei*, 6; *C. parapsilosis*, 4; *Torulopsis glabrata*, 8.

C. albicans was required, fermentation tests were carried out using 3% sugars (dextrose, sucrose, maltose, lactose, galactose, and raffinose). Carbon and nitrogen auxanograms were not determined owing to the time required for their preparation.

RESULTS

On triphenyltetrazolium chloride medium (TTC) *Candida albicans* strains grew easily, producing smooth, raised, glistening creamy-textured colonies, but showed no or limited ability to reduce the

indicator salt, the colonies appearing cream to faintly pink (Fig. 1). Results were frequently determined before chlamydo-spores had appeared in the cornmeal agar, which could be slow (up to four days). If there were a positive result to both tests, the organism was recorded as *C. albicans*. In the absence of chlamydo-spores, but with a positive germ-tube test, strains were also recorded as *C. albicans*. Only very rarely was confirmation by sugar fermentations required.

Candida tropicalis strains usually developed as rather dry, dark, maroon-red colonies on tetrazolium medium. Although the colour was regarded as diagnostic for this species (Table I), the growth rate was variable and fermentation tests were usually carried out in addition. Similarly, other *Candida* species showed evidence of reducing the tetrazolium, the coloured colonies produced ranging from pink to orange; fermentation results were used for confirmation. Only *C. krusei* failed to develop distinctive coloured colonies, but the flat, dry colonies of this species contrasted with the glistening growth of *C. albicans* (Fig. 1).

The incidence of mixed *Candida* species, isolated from the same specimen and not always clearly visible in the original culture plate, occurred in 6% of the total *Candida* isolates (Table II a and b); these mixed isolates were easily detected on the tetrazolium medium. *Candida albicans* occurred as one of the organisms in the majority of mixed infections.

DISCUSSION

GERM TUBES The ability of *C. albicans* to form 'germ tubes' within two to three hours when incubated at 37°C. in certain protein fluids, e.g. human and animal serum (Taschdjian, Burchall, and Kozinn, 1960), plasma, cerebrospinal fluid, and raw egg

TABLE I

TESTS USED FOR IDENTIFICATION OF CANDIDA SPECIES

	Test Medium							
	Serum (germ tubes)	Tetrazolium (TTC) Medium (coloured colonies)	Cornmeal Agar (chlamydo-spores)	3% Sugars (fermentation)				
Period of incubation	2-3 hr	24 hr	24-48 hr	48 hr - several days				
Temperature	37°C	37°C	25°C	37°C				
<i>Candida</i> species				<i>D</i>	<i>M</i>	<i>S</i>	<i>L</i>	<i>Ga</i> <i>Ra</i> <i>Pellicle</i>
<i>C. albicans</i>	+	Cream (glistening)	+	+	+	±	-	± - -
<i>C. stellatoidea</i> ¹	±	Orange-pink	±	+	+	-	-	- - -
<i>C. tropicalis</i>	-	Dark, maroon-red	-	+	+	+	+	± ± ±
<i>C. pseudotropicalis</i>	-	Salmon pink	-	+	+	+	±	± + -
<i>C. krusei</i>	-	White to pale pink (dry)	-	+	-	-	-	- + +
<i>C. parapsilosis</i>	-	Rose pink	-	+	-	±	-	± - -
<i>C. guilliermondii</i>	-	Pink	-	+	-	+	-	± + -

¹Regarded by some as a variant of *C. albicans*

white (Buckley and Van Uden, 1963) is considered to be diagnostic for this *Candida* species alone. Reports have shown a relatively high degree of accuracy in identification with this technique (Mackenzie, 1962; Griffin, 1964). However, in the present survey, approximately 17% of *C. albicans* strains were incorrectly reported as negative by workers inexperienced in this field. This may have been due to failure to produce germ tubes within the specified time, or to the presence of other *Candida* and non-*Candida* species. Of the *C. tropicalis* strains, 50% were reported as producing germ tubes owing to confusion with development of elongated buds. This suggests that the results of germ tube tests used alone are unreliable.

CHLAMYDOSPORE PRODUCTION Seeliger (1955) and Taschdjian (1957) recommended the addition of 1% Tween 80 (a wetting agent) to cornmeal agar as a means of increasing chlamyospore production. We have found a weaker solution (0.02%) to be more satisfactory. Also, angled cuts into cornmeal plates (4.5 mm depth) obviated the necessity for covering the streaks with coverslips or agar overlays (Dawson, 1962) in order to obtain sufficient

anaerobiosis, and gave satisfactory mycelial development and chlamyospore production.

REDUCTION OF TETRAZOLIUM Various indicator compounds, other than tetrazolium salts, have been used in attempts to provide a simple method for detecting and differentiating species of *Candida*; these include bismuth sulphite (Nickerson, 1953), selenite and tellurite (Nickerson, Taber, and Falcone, 1956), and molybdenum (MacLaren and Armen, 1958).

We have found, during the course of routine screening and identifying over 1,500 *Candida* isolates from patients in the past year, that triphenyl-tetrazolium chloride acts as a satisfactory biological indicator when incorporated into a nutrient agar (a slightly fortified Sabouraud's agar). *Candida* species grow as well on this medium as on the routine peptone-dextrose (Sabouraud's) agar. Bacterial growth is inhibited by the addition of neomycin. Agar plates were found to be the most satisfactory for culture of specimens and isolates: many of the early studies were made using prepared tubes or vials¹, which may have accounted for the discrepancies reported.

Many investigators have used incubation temperatures of 25° to 30°C (Pagano *et al.*, 1958; Kutscher, Seguin, Zegarelli, Rankow, Campbell, and Mercadante, 1959a; Kutscher, Seguin, Zegarelli, Rankow, Mercadante, and Piro, 1959b; Hayes and Thompson, 1963; Schmidt, 1966), although Kelly, Kutscher, and Tuoti (1961) found no difference in the results of growth at room temperature and 37°C. We found no necessity to use lower temperatures since sufficient growth and colour changes could be detected after 24 hours at 37°C. Positive readings for *C. albicans* were frequently obtained before chlamydospores were seen on cornmeal agar. Sometimes, cultures on tetrazolium medium gave the

¹Squibbs preparation: approximately 2 ml agar slants.

TABLE IIa

DISTRIBUTION OF CANDIDA SPECIES ISOLATED FROM PATIENTS BETWEEN JULY 1965 AND JULY 1966

	Fulham Hospital	Charing Cross Hospital	Total No.
Total no. of yeast isolates screened	1,276	546	1,822
Total no. of <i>Candida</i> isolates	1,070	451	1,521 (82.5%)
Total no. of yeasts not <i>Candida</i> species (unidentified)	206	95	301 (17.5%)

TABLE IIb

DISTRIBUTION OF CANDIDA SPECIES ISOLATED FROM PATIENTS

<i>Candida</i> Species	Sputum	Main Source of Isolates					
		Throat, Tongue	Vagina	Urine	Stools	Others	Total No.
<i>C. albicans</i>	645	120	260	69	40	42	1176
<i>C. albicans</i> and <i>C. tropicalis</i>	38	2	1	2	1		44
<i>C. albicans</i> and other <i>Candida</i> species	17	5	3	3	2		30
<i>C. tropicalis</i>	101	6	3	5	1	3	119
<i>C. tropicalis</i> and other <i>Candida</i> species	18						18
<i>C. pseudotropicalis</i>	9		2	1			12
<i>C. krusei</i>	21	4	1	5	1		32
<i>C. parapsilosis</i>	12	2	5	1		19	39
<i>C. guilliermondii</i>	2						2
<i>Candida</i> species (unidentified)	34	1	2		2	10	49

only clue as to the identity of the yeast isolate.

We found tetrazolium medium invaluable in checking the identity of 'rough' and 'smooth' colony forms, particularly of *C. albicans* and *C. tropicalis*. Also, in separating mixed *Candida* species, *C. albicans* showed as creamy, smooth colonies which differed from other *Candida* species either in texture or colour, as already described. Non-*Candida* yeasts generally grew poorly on tetrazolium medium and their identity could be checked from growth on cornmeal agar, sugar fermentations, etc.

Clinical specimens (sputum, swabs, urine, etc.) may be cultured direct on to tetrazolium agar plates. Satisfactory isolation and detection of *Candida* species has been described from vaginal specimens (Gillespie, Inmon, and Slater, 1960; Mendel, Haberman, and Hall, 1960) and mucosal and skin scrapings (Rosenthal and Furnari, 1960), though Sinski (1960) found that seven out of 23 yeasts isolated by this method gave false results.

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