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Rapid Methods for Identification of Yeasts

M. HUPPERT,* GLORIA HARPER,[1] SUNG H. SUN, AND V. DELANEROLLE

Mycology Research Department, Veterans Administration Hospital, Long Beach, California 90801

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Opportunistic infections by yeasts have been implicated as one of the major causes of complications in the compromised patient. Rapid recognition and identification of these yeasts is essential for patient management, but conventional liquid medium methods for completing identification tests are cumbersome and time consuming. Rapid tests have been devised based on modifications of methods commonly used in bacteriology. These rapid methods included tests for carbohydrate and nitrate assimilation, fermentation, and urease production. These were compared with several current methods for accuracy of results, for time to final identification, and for economy of time and reagents. In addition, the usual tests for pseudogerm tube formation, for production of hyphae or pseudohyphae, and for growth temperatures were included. The rapid tests achieved 96% or better accuracy compared with expected results, and 46 species of yeasts were identified in 1 to 2 days compared with the 10 to 14 days required by conventional liquid culture methods.

Modern medicine, with the capacity for prolonging the life of a patient, has seen a marked increase in secondary infections among patients whose resistance has beeen compromised by debilitating chronic disease, by therapeutic modalities, and by severe injury. Fungi are a common cause of infections in such patients (7, 9, 11, 12, 14, 16, 19, 24). This increased frequency in the mycoses has been notable in two additional respects: greater severity of the disease and greater diversity in etiological agents (2, 8, 10, 16, 21, 22, 25). Yeasts are among the most common etiological agents.

Since many species of these yeasts had been considered innocuous previously, their recovery from a pathological specimen presents problems for the laboratory which must identify a variety of isolates and for clinicians who must judge their significance. Conventional liquid culture methods for speciation of yeasts are cumbersome and time consuming (15, 25, 27, 28). Rapid methods have been proposed but restrictions in the number of tests used limits identification to relatively few species (1, 5, 6, 8). In addition, most clinical laboratories lack personnel with more than minimal training and experience in identifying fungi.

The present study has been limited to identification of yeasts in the genera *Candida*, *Cryptococcus*, *Torulopsis*, and *Trichosporon*. *Geotrichum candidum*, although not a yeast, has been included because it occurs frequently with yeasts but can be differentiated from the latter by the same procedures. Two objectives were planned. The first involved the development and evaluation of rapid methods for identifying almost all species of yeasts recovered from humans (15). The second required that the methods should be familiar to technologists competent with only bacteriological techniques. Since many of these personnel lack experience in mycology, the tests employed are almost all physiological, and morphological criteria have been limited primarily to observation of whether hyphae (or pseudohyphae) are present or absent. A most essential first step for speciating yeasts is recognition of whether or not perfect stage forms are present, e.g., asci and ascospores. Descriptions of these are beyond the scope of the present study but are available in reference texts (8, 13, 15, 25), or such cultures can be referred to a zymologist for identification. When no information about sexual spore forms is available, all yeast identifications must be considered presumptive.

(This paper includes, in part, a thesis submitted by G. H. to the California State University, Long Beach, in fulfillment of the requirements for the Master of Science degree.)

MATERIALS AND METHODS

The procedures described in this section represent the optimal methods derived from this study. Variations which were studied are presented in later sections.

Strains used. Forty-six previously identified spe-

[1] Present address: Clinical Laboratory, Little Company of Mary Hospital, Torrance, Calif. 90503.

cies (Table 1) were maintained as stock cultures on yeast-malt agar (8, 15).

Preparation of inoculum. Dense suspensions were prepared from confluent growth at room temperature (24 to 48 h) using 6 ml of sterile water per petri dish (100 by 15 mm) followed by transferring the suspension to a sterile tube (16-mm diameter). Although quantitative standardization was performed by optical density (OD) determination and by cell count, a practical and satisfactory estimate of cell density was obtained when the suspension completely obscured the black lines on a standard Wickerham card (3 India ink lines approximately 0.75-mm wide on a white card). The less dense inoculum required for some of the conventional methods employed for comparison with the rapid methods was achieved by diluting the dense inoculum until the black lines were visible as dark bands.

TABLE 1. *List of 46 species studied*

Species	Source[a]
Candida albicans	ATCC 18804
C. ciferrii	CBS 4856
C. curvata	ATCC 10567
C. guilliermondii	ATCC 6260
C. humicola	ATCC 14438
C. intermedia	ATCC 14439
C. kruseii	ATCC 6258
C. lambica	ATCC 9330
C. lipolytica	ATCC 18942
C. lusitaniae	CBS 4413
C. membranaefaciens	CBS 1952
C. norvegensis	CBS 1922
C. parapsilosis	ATCC 22019
C. pelliculosa	Pablo Negroni
C. pseudotropicalis	ATCC 4135
C. ravautii	ATCC 18821
C. rugosa	ATCC 10571
C. silvae	CBS 5498
C. stellatoidea	ATCC 11006
C. tenuis	ATCC 14462
C. tropicalis	ATCC 14056
C. utilis	ATCC 22023
C. zeylanoides	ATCC 10674
Cryptococcus albidus	ATCC 10666
C. gastricus	CBS 1927
C. laurentii	ATCC 18803
C. neoformans	Orda Plunkett M443
C. terreus	ATCC 11799
C. uniguttulatus	CBS 1730
Torulopsis bovina	CBS 2760
T. candida	ATCC 12790
T. etchellsii	ATCC 11504
T. glabrata	ATCC 15545
T. holmii	ATCC 22034
T. inconspicua	ATCC 16783
T. magnoliae	ATCC 13782
T. norvegica	CBS 4239
T. sphaerica	ATCC 2504
T. stellata	ATCC 10673
T. versatilis	ATCC 20222
Trichosporon capitatum	ATCC 10663
T. cutaneum	ATCC 13445
T. inkin	ATCC 18020
T. penicillatum	ATCC 18019
T. pullulans	ATCC 10677
T. variabilis	BNCYC *Endomycopsis chodati*

[a] ATCC, American Type Culture Collection; CBS, Centralbureau voor Schimmelcultures; BNCYC, British National Collection of Yeast Cultures.

Carbohydrate assimilation. The following carbohydrates were used: glucose, maltose, sucrose, inositol, lactose, cellobiose, raffinose, melibiose, erythritol, xylose, galactitol (dulcitol), and trehalose. These were selected on the basis of critical value for differentiating among species (15). The purest grade reagents available were used. For our modified auxanographic method, dry disks containing carbohydrate plus nutrient were prepared. Others have used disks saturated with carbohydrate only (1, 5, 25). A 10× concentration (8.04 g/120 ml of distilled water) of commercially available yeast nitrogen base (YNB) (Difco Laboratories, Detroit, Mich.) was adjusted to pH 6.40 ± 0.05 with 10% NaOH. A 20% solution of each of the 12 carbohydrates was prepared by adding 2 g of carbohydrate to 10 ml of the 10× concentrate of YNB and dissolving with mild heating. Solutions were sterilized by passage through a membrane filter (0.20 μm) followed by aseptic transfer to sterile vaccine vials. A layer of sterile blank 0.25-inch (ca. 0.64-cm) disks (concentration disks, Difco) in petri dishes was saturated with one of the carbohydrate solutions by dropwise addition from a syringe and needle. These were dried 48 to 72 h in an evacuated desiccator containing anhydrous $CaSO_4$. The disks containing inositol, raffinose, and maltose required a second saturation after the first drying period.

The basal agar for the carbohydrate assimilation test contained agar (20 g/1,000 ml), bromothymol blue (0.16 g/1,000 ml), and phosphate buffer (100 ml/1,000 ml). The phosphate buffer stock solution was a 1:1 mixture of 0.067 M Na_2HPO_4 and 0.067 M KH_2PO_4 (pH 6.8). The melted agar was dispensed while hot into 2-oz. (ca. 0.06-liter) screw-cap bottles (approximately 30 ml/bottle). Volumes of 30 to 50 ml can be used, but the smaller volume enhanced the final reading of growth because of the thinner layer of agar. The bottles of agar were autoclaved for 15 min at 121 C. If tightly capped after cooling, these bottles can be stored indefinitely at room temperature.

The assimilation test was performed by pouring one bottle of melted agar for each strain to be tested into sterile petri dishes (150 by 15 mm). After hardening, the plates were streaked for confluent growth with a swab saturated in the dense suspension of cells. The streaked plates were allowed to dry before applying the disks, usually 30 min. Disks were applied to the surface of the agar using a template (Fig. 1) under the dish to insure placing the disks equidistant apart. The position of the glucose disk was marked on the bottom of the petri dish. Plates were incubated at 25 C (room temperature) and read daily for 3 days. The earliest indication of a positive reaction was a change of the indica-

tor color from blue-green to yellow surrounding the disk. This could be confirmed later during the incubation period by enhanced growth of the organism in the area surrounding the disk. Confirmation by reading for growth may be required with those organisms which produce acid from many carbohydrates, turning large sections of the plate completely yellow. Under these circumstances, if no enhanced growth is evident around individual disks, the test is negative for that carbohydrate. This procedure will be referred to as the YNB method in subsequent sections.

The results obtained by the YNB method were compared with those obtained from three additional methods. The Wickerham liquid medium method was performed without agitation according to the procedure described in the Lodder text (15). The oxidation-fermentation (OF) tube method followed the procedures described by Webb et al. (27), except that commercially available OF media (Difco) was used. Tubes in both methods received 0.1 ml of diluted inoculum. The last method used OF media in a petri dish. These were streaked heavily as in the YNB method, and dry disks containing only carbohydrates were placed on the surface. Concentrations of carbohydrates were identical to those used in the YNB method. For the comparative studies, all tests were incubated at room temperature and read at intervals over a 14-day period.

Nitrate assimilation. Dry disks containing a source of nitrogen plus nutrient were prepared in a manner similar to that used for carbohydrate assimilation. A 1% KNO_3 solution in a 4 × concentration of yeast carbon base (Difco) (15, 25, 28) was used to impregnate the disks. Disks for use as a positive control were saturated with a solution of 1% $(NH_4)_2SO_4$ in a 4× concentration of yeast carbon base. Disks for negative controls or persisting endogenous nitrogen source contained only 4× concentration of yeast carbon base. The methods for sterilizing solutions and for saturating and drying the disks were the same as described for carbohydrate assimilation. The basal agar for nitrate assimilation consisted of agar (20 g/1,000 ml) with bromothymol blue (0.08 g/1,000 ml). No phosphate buffer was added to this medium, and adjustment to a light green color (approximately pH 6.8) may be required. The medium was dispensed in approximately 20-ml/bottle amounts and autoclaved for 15 min at 121 C.

Testing for assimilation of nitrate was required for every species according to the scheme developed. One bottle for each strain was melted and poured into sterile petri dishes (100 by 15 mm). Plates were streaked with dense inoculum and dried, and appropriate disks were added in the manner described for carbohydrate assimilation. Incubation was at 25 C for 24 to 48 h. A positive nitrate assimilation test was indicated by a color change from light green to dark blue surrounding the disk containing KNO_3. The color change was caused by the residual, strongly alkaline K^+ after reduction of the NO_3^-. A color change to bright yellow developed around the $(NH_4)_2SO_4$ disk, resulting from utilization of the NH_4^+ and an acid reaction from residual $SO_4^=$ combined with acid from oxidation of the glucose in the yeast carbon base.

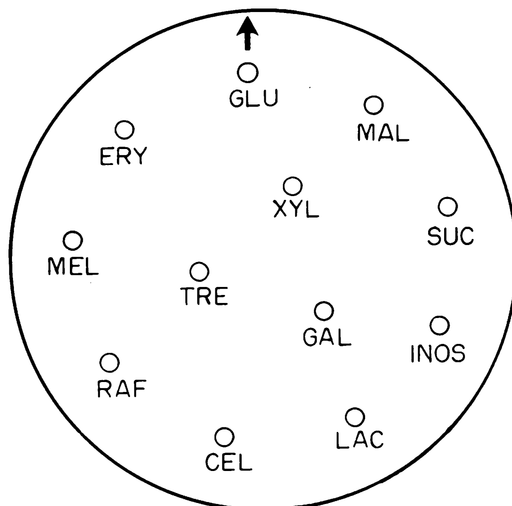

FIG. 1. *Template placed under petri dish for spotting disks and reading results. Abbreviations for carbohydrates (clockwise for outer and inner circles) correspond to sequence in Table 3 beginning with glucose.*

The conventional methods used for comparison were the Delft auxanographic plate (27) and the Wickerham liquid medium procedures (8, 27). Diluted inoculum (0.1 ml) was used, and incubation for both was at room temperature. The Delft plate test was read at 48 and 96 h, and the Wickerham test was read daily for 7 days. A positive by the latter method required subculturing to a second tube to determine whether growth in the first tube could have been caused by carryover of a source of nitrogen.

Fermentation tests. One milliliter of the heavy suspension was placed in each of three 11-mm-diameter test tubes. (The tubes need not be sterile.) Glucose, maltose, and sucrose fermentation tablets (Key Scientific Products, Los Angeles, Calif.) were added one to a tube. One milliliter of molten vaspar was added to each tube carefully with a Pasteur pipette so as not to trap any air bubbles. The vaspar was a mixture (1:1, wt/wt) of petrolatum jelly and paraffin (melting point, 48 to 56 C). The tubes were incubated at 37 C for 24 h. A positive fermentation reaction was indicated by a rise of the vaspar plug, which separated completely from the liquid suspension because of gas production.

Results with this rapid fermentation tests were compared with those obtained by the conventional Durham inverted tube procedure (8, 27). Tubes received 0.1 ml of diluted inoculum and were incubated at room temperature with final readings at 14 days.

Urease production. One milliliter of the heavy suspension was placed in an 11-mm-diameter test tube (nonsterile). One urease tablet (Key Scientific Products) was added, and the tube was incubated at

37 C for 24 h. A bright pink color at the end of the incubation period denoted a positive reaction.

The conventional method used for comparison was Christiansen urease agar slants (8, 27). Slants were inoculated with 0.1 ml of the dense suspension, incubated at room temperature, and read daily for 5 days.

Pseudogerm tube test. Most investigators refer to this as the germ tube test. Mackenzie (17, 18) has stated that the term germ tube "... is a misleading one, as the similarity to the true germinative process of fungal spores is both superficial and transient." He proposed the descriptive term "pseudogerm tubes" for these structures characterized by "...the production (often multiple) from any part of the surface of the parent cell and absence of proximal constrictions..." (17). All strains were studied for pseudogerm tube formation by adding 1 drop of the dense inoculum to 0.5 ml of fetal bovine serum in a test tube (10 by 75 mm) (8). The tubes were incubated at 37 C and read at 3 h for the formation of pseudogerm tubes.

Production of hyphae or pseudohyphae. The presence or absence of hyphae or pseudohyphae was determined in corn meal agar (without glucose) containing 1% Tween 80 (CMT) in a petri dish (50 by 12 mm). The inoculum was obtained from the harvesting plate prior to preparing the dense suspension. A small amount of growth on a stiff needle was pressed through the agar to the bottom of the plate and then drawn through the agar at an angle to make a long cut. Incubation was at room temperature for 24 to 48 h. Production of hyphae was observed by inverting the plate on the stage of the microscope and viewing with the low-power (10×) objective lens.

Additional tests. For some species, temperature tolerance (i.e., growth at 20 and 37 C) was required for identification. The 20 C tolerance was observed using the yeast-malt agar plate prior to harvesting. An additional plate of yeast-malt agar was incubated at 37 C when this information was needed for final identification. If morphology was required to differentiate among several species, microscopic examination was made on the surface of the CMT plate.

RESULTS

Density of suspension. Since rapid results with these new procedures required a heavy inoculum, it was necessary to determine the upper and lower limits of inoculum density for obtaining accurate and reproducible results. The wavelength (Coleman junior spectrophotometer, model 6D) yielding maximum sensitivity for detecting a difference in density of cells was determined using two dilutions of selected species from the genera *Candida*, *Cryptococcus*, *Torulopsis*, and *Trichosporon*. The ODs for these dilutions were read at several wavelengths in a scan of the visible spectrum. The wavelength yielding the greatest difference between the two curves was chosen as the one providing maximum sensitivity for these yeast cell suspensions. A typical example appears in Fig. 2. The wavelengths of maximum sensitivity for all species clustered around 580 nm. Upper limit density studies were performed by harvesting successive plates of the several species with the initial 6 ml of water until the suspension attained a viscosity precluding the harvesting of additional plates. This and several serial dilutions were used to perform the carbohydrate and nitrate assimilation, urease production, and fermentation tests. Accurate, reproducible, and rapid results were obtained with all suspensions yielding an OD ≥ 0.6 (approximately 10^8 cells/ml), and it was concluded that only a minimum limit for inoculum density was required. A suspension in a 16-mm-diameter tube which obscures the lines on a Wickerham card has an OD ≥ 0.6, and this simple card method was used routinely.

Carbohydrate assimilation. The four methods were evaluated in terms of percentage of agreement with expected reactions for each species as published in Lodder (15), referred to as the "reference." The combined results for all 46 species with each of the four methods are pre-

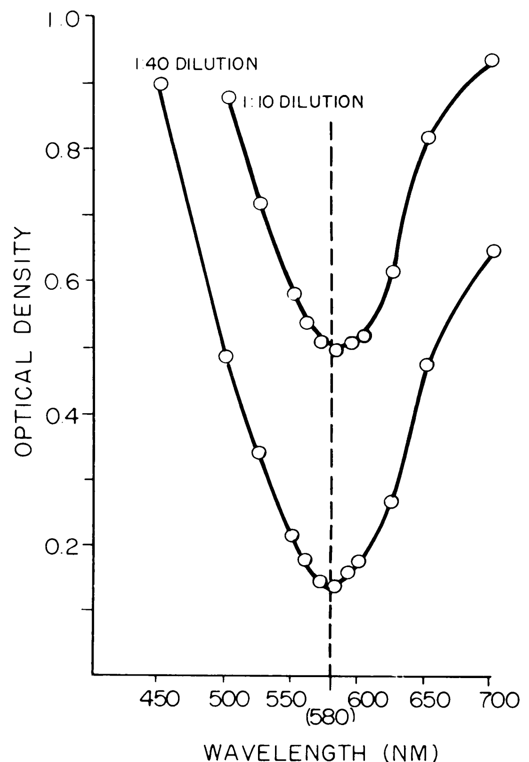

FIG. 2. *Typical example for determining the wavelength which produced the maximum sensitivity for detecting differences in density of cells in a suspension. Species was C. guilliermondii.*

sented in Table 2 and Fig. 3. The rapid YNB method produced 90% of the expected results within 24 h and 97% agreement with the reference by 48 h. The Wickerham method required at least 3 to 5 days to attain the same level of agreement as the YNB procedure, although the former achieved 100% agreement by 14 days. This was expected since the results published in the reference were obtained with the Wickerham method. The two procedures using OF media were not as good. The OF plate method was particularly unsatisfactory because color changes began to revert on day 3 from yellow back to blue as the carbohydrate was exhausted, peptone was utilized as a carbon source, and the medium became alkaline. Adams and Cooper (1) reported similar difficulties with OF media. The data in Table 2 and

TABLE 2. *Comparison of results obtained by four methods for assimilation of carbohydrates*

Method	Incubation time (days)			
	1	2	3–5	6–14
OF, plate	82 ± 13[a]	86 ± 15	86 ± 15	86 ± 15
OF, tube	83 ± 11	89 ± 9	93 ± 5	93 ± 5
YNB	90 ± 7	97 ± 2	98 ± 2	98 ± 2
Wickerham	78 ± 11	88 ± 10	98 ± 3	100 ± 1

[a] Results are presented as percentage of agreement with reference (15) ± standard deviation.

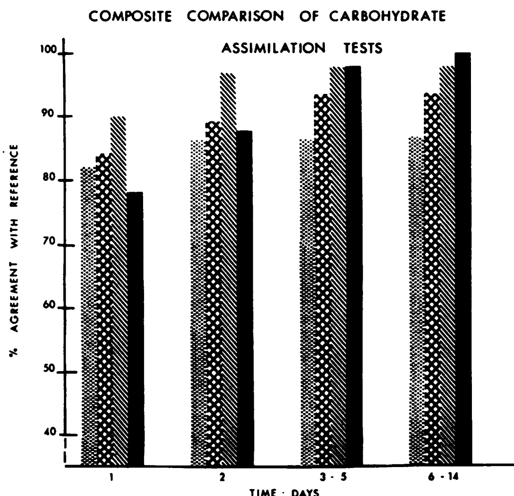

FIG. 3. *Comparison of composite results obtained by four methods for assimilation of carbohydrates expressed as percentage of agreement, with expected results as published in reference 15. Symbols for bars: dotted, OF plate method; cross-hatched, OF tube method; striped, YNB rapid method; solid, Wickerham method.*

Fig. 3 do not reflect this reversion for the 3- to 5-day and 6- to 14-day periods, but only that a maximum of 86% agreement had been obtainable by this method. In preliminary studies YNB had been used in the medium in plates rather than in the disks which contained only the carbohydrates. The same reversion of indicator color occurred, and this was discarded in favor of incorporating nutrient and carbohydrate in disks. In the latter method, reversion of indicator color did not occur (Fig. 4).

The YNB method was less variable during the first 2 days than the other methods (Table 2). The principal cause for this variability is illustrated in Fig. 5–7 for the three carbohydrates trehalose, cellobiose, and raffinose. The percentage of agreement with the reference after 24 h is relatively low for all three of these carbohydrates by each of the four methods. By 48 h, however, the YNB method achieved 96% or better agreement with these three carbohydrates, whereas the other methods attained only 82% at best by this time. These results indicate that factors which presented problems for the conventional liquid media methods had relatively little effect on the rapid YNB method for carbohydrate assimilation.

Fermentation tests. The rapid method and conventional Durham tube fermentation tests were evaluated in terms of the published expected results (15) for each species. The rapid test (Fig. 8) produced accurate results in 1 day with 94% or better of the expected reactions, whereas the conventional Durham tube test required 14 days for comparable agreement. Two-thirds of the 6% incidence of nonagreement using glucose in the rapid test were with species which yielded positive reactions when a negative test was expected. This may have resulted from incubation at 37 C with the rapid procedure, since the reactions reported in the reference were based on incubation at 25 to 28 C for 24 days. In addition to yielding results quickly, the rapid test was very easy to read. When fermentation had occurred, the vaspar plug had been raised several centimeters in almost every instance.

Nitrate assimilation. A comparison of results obtained by the rapid method and by the Delft and Wickerham procedures indicated 100% agreement with the reference by all three methods. Incubation time for the YNB method was significantly shorter, 1 to 2 days versus 4 to 14 days. The change in indicator color from light green to dark blue around the KNO_3 disk was obvious and dramatic with all species assimilating nitrate, but either no change or a slight yellowing occurred with species which were negative for nitrate assimilation. The disk contain-

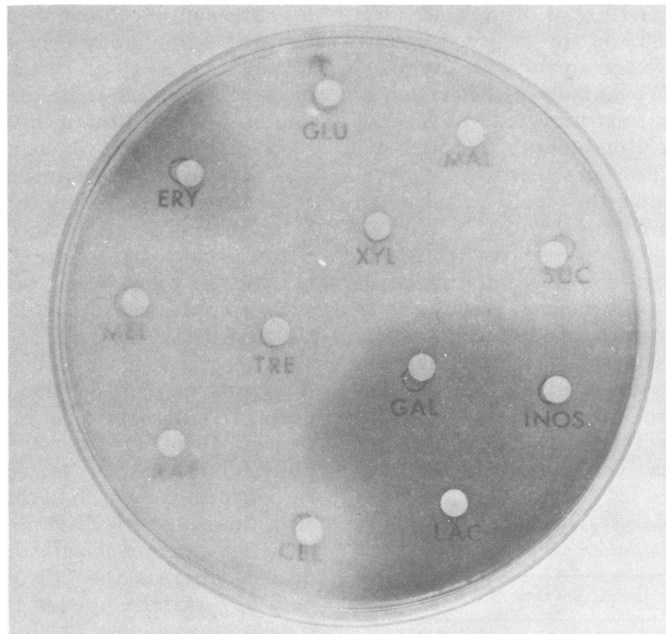

FIG. 4. *Typical example of carbohydrate assimilation results by the YNB rapid method (C. guilliermondii at 18 h).*

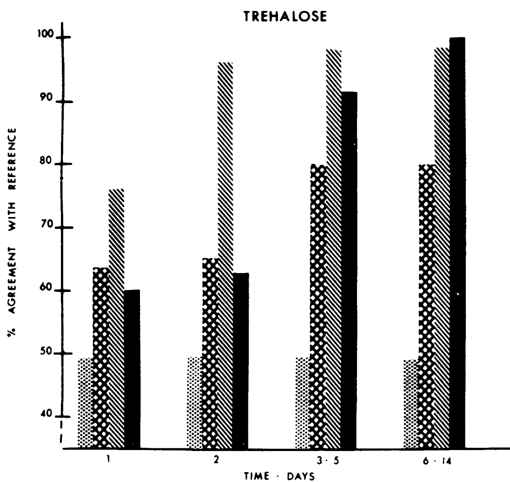

FIG. 5. *Results obtained by four methods for assimilation of trehalose. (See Fig. 3 for key to bar graphs.)*

ing $(NH_4)_2SO_4$ was surrounded by a yellow zone with all species, and the area around disks containing yeast carbon base without a source of nitrogen either remained light green or showed a slight change to yellow. Therefore, carryover of endogenous nitrogen did not influence results with the rapid test, and there was no need for starving cells or subculturing. In fact, the medium control disk was not required.

Pseudogerm tube production. As expected, *C. albicans* and *C. stellatoidea* produced pseudogerm tubes consistently within the 3-h incubation period. However, *C. tropicalis*, *C. parapsilosis*, and *Cryptococcus gastricus* also produced structures which resembled pseudogerm tubes. Although these were elongated blastospores, personnel inexperienced with the morphology of yeasts might not recognize the difference and an incorrect identification could re-

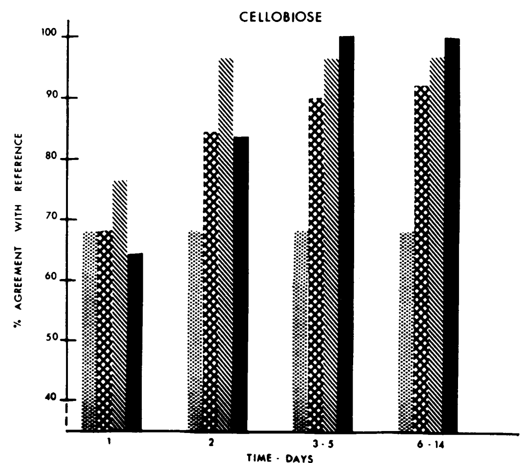

FIG. 6. *Results obtained by four methods for assimilation of cellobiose. (See Fig. 3 for key to bar graph.)*

sult. In our opinion complete reliance on the pseudogerm tube test for identifying *C. albicans* or *C. stellatoidea* by inexperienced personnel may not be justified (see Discussion).

Hyphae or pseudohyphae formation. All species in the genera *Cryptococcus* and *Torulopsis* failed to form any structures resembling hyphal formation in the CMT agar. In contrast, all *Candida* and *Trichosporon* species and the strain of *G. candidum* were positive. We recognize that occasional strains of *C. guilliermondii* and *C. parapsilosis* may not produce

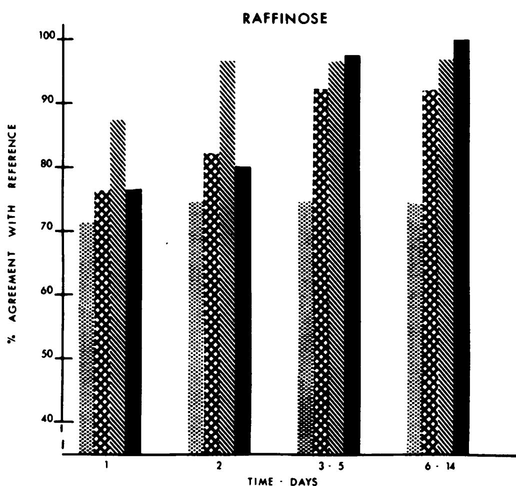

FIG. 7. *Results obtained by four methods for assimilation of raffinose. (See Fig. 3 for key to bar graphs.)*

obvious hyphal structures, and this has been considered in the construction of our schema for identification of these yeasts (see below).

Urease production. A comparison of results obtained by the rapid method and by the conventional method showed 100% agreement with the reference by both methods. The principal advantage of the rapid method was the much shorter incubation time, i.e., 1 day versus 5 days.

Quality controls for assimilation, urease, and fermentation tests. Satisfactory quality control for these tests required a suspension yielding positive reactions and one resulting in negative tests. No single species produced positive results in all tests. Several strains in combination were tried, and the best results were obtained with *C. pelliculosa* and *Cryptococcus laurentii*. Each species was grown and harvested separately and then tested for satisfactory inoculum density. Equal aliquots of each suspension were mixed, and the mixture was used to perform the rapid procedures. All the rapid tests were definitely positive within 48 h. If preferred, suspensions of these two species could be used separately rather than mixed into a single suspension. Our strain of *Cryptococcus laurentii* assimilated all 12 carbohydrates and was urease positive. The *C. pelliculosa* strain produced positive reactions in tests for nitrate assimilation and fermentation. A suspension of *Trichosporon capitatum* was used as a negative control. Our strain of this species assimilated only glucose and was negative in all other tests.

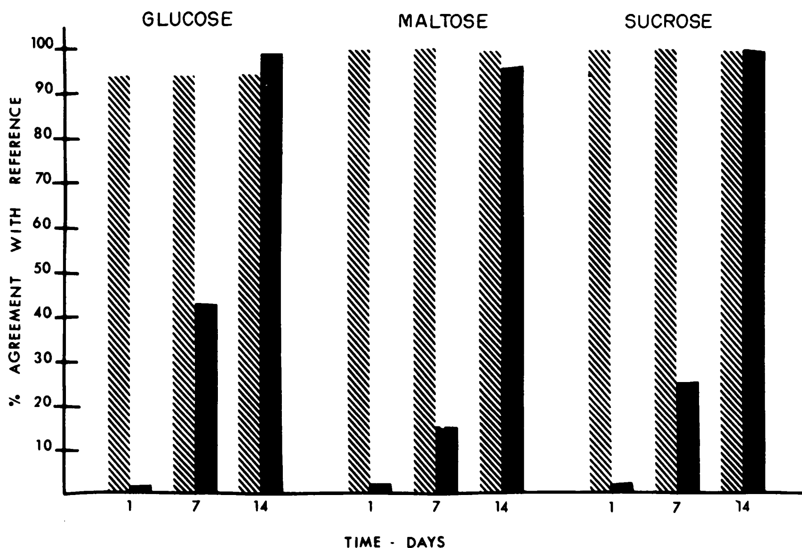

FIG. 8. *Comparison of results obtained by the rapid and by the Durham tube fermentation tests expressed as percentage of agreement, with expected results as published in reference 15. Symbols: striped bar, rapid method; solid bar, Durham tube method.*

Schema for identification of yeasts. This series of tests would not be complete without presenting a schema by which the results could be used easily for identification of these imperfect yeasts. A schema has been constructed for this purpose (Table 3). It includes all species in the genera *Candida, Cryptococcus, Rhodotorula, Torulopsis,* and *Trichosporon* which, according to Lodder (15), have been recovered from humans or animals. In addition, *Saccharomyces cerevisiae* and *G. candidum* (not a yeast) have been included because of the need to distinguish these common fungi from species in the above five genera.

The principal objective guiding the construction of this schema was to include only those reactions which were most important for differentiating among species, keeping in mind that many personnel in clinical laboratories have little experience with these organisms. A primary separation was made on the basis of whether hyphae or pseudohyphae were either well developed or absent (or rudimentary) as seen in the CMT agar. The two species of *Candida* (*C. guilliermondii* and *C. parapsilosis*), in which occasional strains fail to produce pseudohyphae, appear under both categories. Similarly, occasional strains of *R. glutinis* and *R. rubra* may produce pseudohyphae, and these appear in both sections of the schema. Species with variable reactions in some tests appear several times, when the result with a single reagent might be positive and again when it would be negative. Therefore, decisions in almost all cases were reduced to simply positive or negative results. Groups of species were formed on the basis of assimilation tests for nitrate, glucose, maltose, and sucrose. These groups could be differentiated into species by reading results for only those additional tests which were critical within the group. Preliminary trials with this schema by technologists unfamiliar with the yeasts have met with general acceptance and have resulted in successful species identifications of yeasts previously reported incompletely, e.g., unidentified yeasts or *Candida* species that are not *C. albicans*. An additional benefit is gained by including only critical reactions in the schema. Almost all the reactions responsible for the lack of agreement between the rapid methods and the reference have been eliminated.

DISCUSSION

Achievements by modern medical science have produced a population of people uniquely susceptible to infection by microorganisms previously considered innocuous. Some of these organisms exist saprophytically in man's environment, whereas others occur comensally, and the individual with compromised defenses cannot escape eventual exposure. The yeasts, as a group, are involved frequently in these opportunistic infections.

The true incidence of secondary infections caused by yeasts is difficult to assess since, undoubtedly, many of these have not been recognized. Quie and Chilgren (23) have stated that "only 30% of disseminated candida infections are diagnosed correctly antemortem so that improved methods of diagnosis are clearly needed." There is ample evidence, however, that not only morbidity but also mortality must be significant. Law, MacMillan, and their associates (14, 19) reported studies on 427 burn patients. *Candida* species were recovered one or more times from 63.5% of the patients. In addition to positive cultures from the wound, *Candida* species were found in urine (48%) and in blood (5%), and, of the 65 patients who died, 14 (22%) deaths were attributable to disseminated candidiasis. Miller et al. (20) reported that in the Center for the Study of Trauma at the University of Maryland 16% of the nosocomial infections were caused by *Candida* species, exceeded only by *Pseudomonas* infections (24%). These were primarily shock trauma and postoperative thoracic surgery patients, with only an insignificant number of burn cases. Gaines and Remington (7) reviewed surgical records for the 1960–1967 period and found 42 cases developing systemic candidiasis, with 19 deaths. They pointed out that candidiasis was not diagnosed early enough for appropriate treatment in more than 50%. Rifkind et al. (24) described 107 cases receiving renal transplants, with 51 deaths. Systemic mycoses were involved in 23 (45%), *Candida* species accounting for 14. Hutter and Collins (9) found 202 mycoses in cancer patients, with yeasts responsible for 130. Eighty-four of the latter occurred in the leukemia-lymphoma group. Kahanpää (10), in an extensive survey involving 8,290 specimens, identified a total of 35 species of yeasts in 21% of bronchial secretions, 34% of lung tissue specimens, 71% of autopsy lung specimens, and 20% of the pleural effusions. One must conclude that yeasts represent a significant problem in these diseases and that early recognition is essential to insure appropriate therapy.

Our approach to the development of methods for identifying yeasts has recognized these requirements. The procedures are adapted almost entirely from those familiar to microbiologists with little experience in mycology, and the results are obtained rapidly, usually in 2 to 4 days

after primary isolation compared to 10 to 14 days by conventional liquid culture methods. In addition, the presumptive identification scheme has been expanded to 62 separate species compared to the more common 25, or fewer, species included in most schemes (8, 25). In preliminary trials, these rapid methods have proven to be readily adaptable to the clinical laboratory routine. Almost all of the species identifications are accomplished with the assimilation, fermentation, and urease tests after determining that no sexual forms are present (asporogenous yeasts) and after recognition of whether or not hyphal structures are produced. There are only four instances requiring differentiation by growth at 20 or 37 C (*Cryptococcus neoformans* versus *Cryptococcus luteolus*; *T. sphaerica* versus *T. candida*; *T. bovina* versus *T. pintolopesii*; *C. krusei* versus *C. slooffii*), and these can be resolved without subculturing. For example, with *Cryptococcus neoformans* and *Cryptococcus luteolus*, the fermentation tests are incubated at 37 C and *Cryptococcus neoformans* produces acid (no gas) from glucose, whereas *Cryptococcus luteolus* would show no change in the color indicator since it does not grow at 37 C. Similarly, with *C. krusei* and *C. slooffii*, the former would be positive for glucose assimilation at room temperature whereas the latter would be negative, although it would produce gas from glucose at 37 C. A requirement for differentiation by additional morphological studies occurs in only five cases, and in at least two of these the additional studies might not be necessary; i.e., *C. humicola* is always urease positive whereas *Trichosporon cutaneum* is variable, and *C. lambica* always produces gas from glucose whereas *Trichosporon penicillatum* usually is negative.

The YNB method possesses several advantages over the Wickerham method in addition to yielding results more quickly. The color change occurred very early (usually after overnight incubation) and was an added aid in obtaining rapid results. Adams and Cooper (1) reported that the color change in their method was "...synonymous with growth..."in every test completed at that time. Furthermore, growth could be read quite easily by 48 h as seen in Fig. 4, where the printing on the underlying template is sharp at disks where growth did not occur but hazy at disks where growth was present. Obviously, any carryover of a source of carbon with the inoculum is not a problem with the YNB method, since very little, if any, growth occurred around disks containing a carbohydrate which could not be utilized by the species tested. Additional advantages of the YNB method are economy and rapid preparation once disks have been prepared and stored. A single plate is used compared to 12 tubes for the Wickerham method, and these plates can be prepared in one-half the time required for inoculation of the 12 tubes in the Wickerham method.

At this point we do not know how strains with latent assimilation reactions according to Lodder (15) will behave when a very dense inoculum is used. Allowance for this has been provided in the present scheme for identification (Table 3) by including the species twice, once for a negative reaction and again for a positive reaction (e.g., *Cryptococcus gastricus* for lactose assimilation).

There are several points which should be noted with respect to the nitrate assimilation and fermentation tests. The nutrient used in the nitrate test is acid, and during the first few hours of incubation the area of basal medium surrounding the disks may change from a light green color to pale yellow. Adding buffer to the basal medium eliminated this but it also reduced the sensitivity of the test, resulting in some false-negative reactions. This pale yellow coloration is not apparent after overnight incubation and it has not influenced the results reported above. One should be aware, however, that this early change in color does not indicate that growth has occurred. A similar early color change may occur around disks in the carbohydrate assimilation test, but this too does not affect readings after overnight incubation. The fermentation tests require the careful overlay of vaspar on the cell suspension to avoid trapping air between the two. Since these tubes are incubated at 37 C after preparation at room temperature, trapped air will expand and might lead to a false reading as a bubble of gas. If trapped air is noted after addition of the vaspar layer, the position can be marked on the tube, and the test should be read as positive only if the vaspar plug is displaced more than would be expected from expansion of the trapped air. This has not been a serious problem because the plug is displaced at least several centimeters in almost all cases of gas formation. The vaspar must be maintained completely melted and should not solidify until after being layered on top of the cell suspension. This will insure a full seal by the vaspar. We have maintained the bottle of vaspar in a hot water bath while preparing tests.

The pseudogerm tube test is an excellent procedure for presumptive identification of an isolate as either *C. albicans* or *C. stellatoidea*, but interpretation of this test by inexperienced tech-

TABLE 3. *Schema for identification of yeasts found on humans and warm blooded animals*[a]

Hyphae or pseudohyphae production	Basic assimilations				Species	Differential assimilations									Gas from			Urease	Growth at		Other
	Nitrate	Glucose	Maltose	Sucrose		Inositol	Lactose	Cellobiose	Raffinose	Melibiose	Erythritol	Xylose	Galactitol	Trehalose	Glucose	Maltose	Sucrose		20 C	37 C	
Absent or rudimentary					*Cr. albidus*	+									0			+			
		+	+	+	*T. versatilis*	0									+			0			
					R. glutinis	0									0			+			
					Cr. terreus	+												+			
	+	+	+	0	*T. versatilis*	0								+				0			
					T. etchellsii	0								0				0			
		+	0	+	*T. magnoliae*													0			
		+	0	0	*Cr. terreus*	+												+			
					T. norvegica	0												0			
					Cr. laurentii	+	+		+	+	+							+			
					Cr. gastricus	+	+		0	0	0							+			
					Cr. neoformans	+	0						+					+	+	+	
					Cr. luteolus	+	0						+					+	+	0	
					Cr. uniguttulatus	+	0						0					+			Sucrose assimilation strong
					Cr. gastricus	+	0						0					+			Sucrose assimilation weak
		+	+	+	*T. sphaerica*	0	+	+							+			0	+	0/+	
					T. candida	0	+	+							0/+			0	+	+	
					T. candida	0	0	+							0/+			0			
					R. rubra	0	0	+							0			+			
					C. guilliermondii	0	0	+							+			0			
	0				*R. rubra*	0	0	0	+						0			+			
					S. cerevisiae	0	0	0	+						+			0			
					C. parapsilosis	0	0	0	0						+			0			
		+	+	0	*Cr. gastricus*	+												+			
					R. minuta	0	+								0			0			
					T. sphaerica	0	+								+			0			
		+	0	+	*T. holmii*	0	0							+	+			0			
					T. stellata	0	0							0	+			0			
					R. minuta	0	0							+	0			0			

					T. glabrata	0								+	+			0			
		+	0	0	*T. bovina*	0								0	+			0	+	+	
					T. pintolopesii	0								0	+			0	0	+	
					T. inconspicua	0								0	0			0			
Well developed					*Tr. pullulans*	+	+				+				0			+			
					Tr. pullulans	0	+				+				0			+			
	+	+	+	+	*C. pelliculosa*	0	0				+				+			0			
					C. utilis	0	0				0				+			0			
					R. glutinis	0	0				0				0			+			
					C. humicola	+	+			+								+			No arthrospores
					Tr. cutaneum	+	+			+								V			Arthrospores
					Tr. cutaneum	+	+			0											Arthrospores
					C. curvata	+	+			0											No arthrospores
					Tr. inkin	+	+			0											Arthrospores; *Sarcina*-like packets present
					C. ciferrii	+	0			+											
					C. humicola	0	+		V	+					0			+			No arthrospores
					Tr. cutaneum	0	+		V	+					0			V			Arthrospores
					C. tenuis	0	+		+	0					+						
					C. curvata	0	+		+	0					0						No arthrospores
					Tr. cutaneum	0	+		V	0					0						Arthrospores
					C. intermedia	0	+		0	0					+						
		+	+	+	*C. membranaefaciens*	0	0	+	+	+	+										
					C. guilliermondii	0	0	+	+	+	0										
					Tr. variabile	0	0	+	+	0	+										
					R. rubra	0	0	+	+	0	0										
					C. tropicalis	0	0	+	0	0	0				+	+	+				
					C. viswanathii	0	0	+	0	0	0				+	+	0				
					C. lusitaniae	0	0	+	0	0	0				+	0	V				
					Tr. variabile	0	0	0	+	0	+										
					R. rubra	0	0	0	+	0	0				0						
					S. cerevisiae	0	0	0	+	0	0				+						
					C. tropicalis	0	0	0	0	0	0				+	+	+				
	0				*C. albicans*	0	0	0	0	0	0				+	+	0				
					C. parapsilosis	0	0	0	0	0	0				+	0	0				
					Tr. cutaneum	+	+								0						
		+	+	0	*Tr. cutaneum*	0	+								0						
					C. stellatoidea	0	0								+	+					
					C. ravautii	0	0								0/+	0					

TABLE 3—*Continued*

Hyphae or pseudo-hyphae production	Basic assimilations				Species	Differential assimilations									Gas from			Urease	Growth at		Other
	Nitrate	Glucose	Maltose	Sucrose		Inositol	Lactose	Cellobiose	Raffinose	Melibiose	Erythritol	Xylose	Galactitol	Trehalose	Glucose	Maltose	Sucrose		20 C	37 C	
					Tr. cutaneum	+	+								0						
		+	0	+	*Tr. cutaneum*	0	+								0						
					C. pseudotropicalis	0	+								+						
					Tr. cutaneum	+	+	+/0						V							
					Tr. cutaneum	0	+	+/0						V							
					C. lipolytica	0	0	+			+			0	0						
					C. norvegensis	0	0	+			0			0	+/0						
					C. lipolytica	0	0	0			+			0	0						
					C. lambica	0	0	0			0	+		0	+						No arthrospores
		+	0	0	*Tr. penicillatum*	0	0	0			0	+		0	0/+						Arthrospores & blastospores
					G. candidum[b]	0	0	0			0	+		0	0						Arthrospores; no blastospores
					C. rugosa	0	0	0			0	+		0	0						No arthrospores
					C. zeylanoides	0	0	0			0	0		+	0/+						
					C. krusei	0	0	0			0	0		0	+				+	+	
					C. slooffii	0	0	0			0	0		0	+				0	+	
					Tr. capitatum	0	0	0			0	0		0	0						Arthrospores
					C. silvae	0	0	0			0	0		0	0/+						No arthrospores

[a] Symbols are: +, positive; 0, negative; +/0, usually positive; 0/+, usually negative; V, either positive or negative. Abbreviations for genera are: *C.*, *Candida*; *Cr.*, *Cryptococcus*; *G.*, *Geotrichum*; *R.*, *Rhodotorula*; *S.*, *Saccharomyces*; *Tr.*, *Trichosphoron*.

[b] *G. candidum*, although not a yeast, is frequently isolated along with yeasts.

nologists requires some caution. Dolan (5) reported negative results in the original test for 17 of 133 (13%) strains of *C. albicans*. After identification of species by additional tests, these strains were found to form relatively few pseudogerm tubes which were missed initially. Our experience has been similar. In addition to this potential for false-negative results, one must reckon with the possibility for obtaining false-positive readings. Ahearn (2) has commented that "inexperienced personnel may mistake germinating arthrospores of *G. candidum* or *Trichosporon* species for germ tubes of *C. albicans*." He points out also that among species of yeasts which can produce pseudohyphae ". . . colonies may have formed hyphal elements on isolation agar. These may be mistaken for germ tubes when observed in serum." Buckley and van Uden (2) also noted that some pseudomycelium might be carried over in the initial inoculum, although they were able to distinguish this from true pseudogerm tube formation. We agree with these observations that inexperienced personnel may obtain false-positive results since we have seen even *Cryptococcus gastricus* produce elongated (hyphal-like) blastospores still attached to oval mother cells. In one of our laboratories *C. tropicalis* was isolated repeatedly from the blood and urine of a patient who had open heart surgery. This strain of *C. tropicalis* produced elongated pseudohyphal cells in serum within 3 h and these were grossly similar to pseudogerm tubes. One must recognize the possibility that this may occur more frequently as the identification of yeasts involved in opportunistic infections becomes more precise. Unfortunately, there is evidence that some laboratories rely exclusively on this test for identification of *C. albicans* (14, 20), and this cannot be justified. In our opinion, a positive pseudogerm tube test can be considered presumptive identification of *C. albicans* or *C. stellatoidea* to be confirmed by additional tests, whereas a negative test does not exclude the identification of these two yeasts.

The sequence to be followed for identification of yeasts is readily adaptable for use in clinical microbiology (Fig. 9). Since prepared disks and basal media can be stored, the tests can be set up rapidly. In our experience, disks stored in a desiccator have been satisfactory for up to 6 months, with the exception of xylose. When the xylose disk turns brown, it is no longer reliable. There is some advantage to performing the test for hyphae or pseudohyphae directly from several isolated colonies on the primary isolation media to determine whether more than one species is present. One more day would be required, but additional information would be

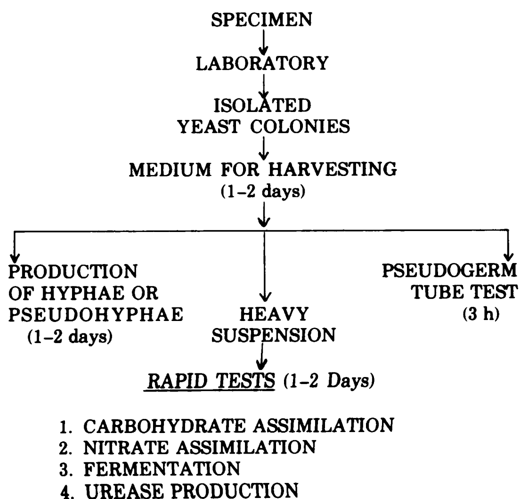

FIG. 9. *Sequence of procedures for identification of yeasts.*

obtained in some cases. The inoculum for the harvesting medium could be obtained from the surface of the CMT agar plate since it has not been covered with a glass cover slip.

Our schema is the same in principle as that reported by Barnett and Pankhurst (3), i.e., identification of asporogenous yeasts based primarily on physiological tests and multiple entries of a species when results of a test are variable. These authors also used the data published by Lodder (15) for 341 species and added descriptions of 93 new species for a total of 434. Their key to the identification of these yeasts was constructed after computer analysis revealed the most efficient tests for differentiation among species. This publication is recommended for those laboratories interested in an expanded schema for identification of asporogenous yeasts.

We recognize that a search for, and identification of, yeasts with all specimens is not practical for clinical laboratories with workloads already strained. Communication and cooperation between physician and microbiologist is required to identify cases in which recovery of yeasts would bear significantly on clinical management of the patient. The need for this collaboration has been expressed clearly by Klainer and Beisel (12): "In this situation, a close reciprocal relationship must develop between the clinician at the bedside and the microbiologist in the laboratory. The physician must alert the microbiologist to the possibility of an opportunistic infection; in return, the microbiologist must regard isolation by culture of 'contaminants' or saprophytes, especially if recurring, with caution and suspicion. Together, such a

medical team must attempt to determine the significance of the presence of these organisms."

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