Proceedings of the Iowa Academy of Science

Volume 81 | Number Article 9

1974

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WALKER, LEILA J., and MARGUERITE R. LUECKE (Laboratory Service, Research Service, Veterans Administration, Wadsworth Hospital Center, Los Angeles, California 90073). The Identification of Yeasts from Clinical Material. *Proc. Iowa Acad. Sci.* 81(1): 14-22, 1974.

A workable, practical scheme for the identification of yeasts isolated from clinical specimens is presented. Relationships between medically important sexual stages and imperfect forms, and characteristics of the sexual stages in clinical material, are described.

Included in this report is a guide to yeast identification which relies on the Luecke plate, a modified Dalmau plate.

INDEX DESCRIPTORS: Yeast Identification, Non-Filamentous Fungi, Mycology in Medicine.

Medical mycology is presently emphasizing sophisticated methods for isolation and identification of yeasts from clinical material. Many factors have contributed to this trend toward sophistication, including an increase in the numbers and kinds of isolated yeasts which are unfamiliar to the clinical mycologist. Currently approximately 70 species in 10 genera are recognized (Lodder, 1970). These yeasts include species of *Rhodotorula* (Lodder, 1970; Louria et al., 1967; and Blair et al., 1970), *Kluyveromyces* (Lodder, 1970), *Saccharomyces* (Lodder, 1970), *Hansenula* (Lodder, 1970), *Pichia* (Lodder, 1970), *Debaryomyces* (Lodder, 1970), *Sporobolomyces* (Lodder, 1970), and 20 species of *Candida* (Haley, 1971; Lodder, 1970; and Painter and Isenberg, 1973). Other examples include species of *Cryptococcus* other than *neoformans* (Blair et al., 1970; Haley, 1971), including a red *infirmo-miniatus* (Lodder, 1970) and an unusual species of *Torulopsis* (Haley, 1971; Lodder, 1970). These unfamiliar isolates may be caused by new drugs, drug combinations, or underlying diseases. Whatever the cause, more saprophytic yeasts are associated with clinical material and/or disease processes than before.

Not only is the clinical mycologist currently concerned with the isolation of more yeasts with opportunistic potential, but also with a growing trend toward dual infections, primarily yeast and bacterial. Dual infections have been seen in Wadsworth V.A. patients undergoing home dialysis.

In addition to unfamiliar species, the clinical mycology laboratory discovers occasional aberrant yeast isolates, possibly produced by certain drugs, which only the well-trained mycologist may recognize. Experiences with atypical fungi revealed a *C. neoformans* without a capsule, a *Geotrichum* sp. that formed atypical arthrospores, and strains of *C. albicans* that failed to form typical chlamydospores on corn meal tween agar.

All of the above-mentioned problems indicate that a clinical mycology laboratory interested in species identification must be adequately equipped to isolate and identify well-known as well as unusual yeasts. Although the physician determines the significance of the various yeasts isolated, the clinical mycologist aids in this regard by designating yeast genera and species present, and counting the yeast colonies present in the initial isolate. The consistent reporting of large quantities of one particular species is significant to the physician.

Certain guidelines are necessary to help the clinical mycologist classify the ever-expanding group of yeasts present in clinical material, to deal with morphological aberrations, and to facilitate laboratory studies necessary for yeast identification. Such studies are necessary to ascertain the range of variation in the common yeasts, such as *Trichosporon, Cryptococcus, Candida,* and *Torulopsis,* as well as those expected to be isolated with greater frequency as our identification procedures improve, such as *Rhodotorula, Saccharomyces, Cryptococcus* species other than *neoformans, Candida* species other than *albicans* and *tropicalis,* and the following perfect stages: *Debaryomyces, Kluyveromyces, Pichia, Hansenula,* and *Sporobolomyces.*

It is the purpose of this report to present materials for the identification of all yeasts associated with clinical specimens and/or diseases. These materials were prepared as a result of a three-year study (1970-1973) of over 3,400 yeasts isolated at the Wadsworth V.A. Hospital clinical mycology laboratory. They include quality control measures and other important steps required for accurate yeast identification, and a description of the sexual stages of some yeasts. The enclosed scheme (Figure 3) involves the yeasts identified in our three-year study and those cited by Lodder (1970), Dolan (1971), and Haley (1971).

METHODS

Initial Examination of the Isolates. The morphology of all yeast-like growth is noted and morphologically different colonies are examined microscopically. Whether the isolate is pure or mixed, the technique for microscopic examination of yeast size and shape is that used by Haley (1971). A thin preparation of yeast in water permits rapid settling of the organism on a slide and allows immediate examination.

Morphologically different colonies often reveal different sizes and shapes of yeast cells upon microscopic examination, while pure colonies reveal similar sizes and shapes. But morphologic and microscopic examination alone do not indicate purity. Plating methods are necessary to ascertain the purity of cultures.

Plating Method for Pure Cultures. A yeast may be accompanied by bacteria, other yeasts, and/or filamentous fungi in

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the initial isolate. The yeast must be isolated and purified. The same emulsified portion of the yeast colony in sterile distilled water which was used for direct microscopic examination may be used here. A loopful of this emulsified specimen is streaked on Bacto Sabouraud dextrose agar and incubated at room temperature to obtain isolated colonies. A yeast suspension in water separates the individual yeast cells even before streaking, thus allowing more complete isolations. If the culture is mixed, different colony types will be seen in the area where single colonies are separated. Each different colony should undergo a second streaking on Sabouraud's agar as a purity check (Haley, 1971).

Pure cultures are maintained on Bacto yeast malt agar (YM) or Bacto malt agar (MA), but before various methods for identification are utilized, each pure colony is subcultured to YM agar. If possible two successive transfers to YM agar are made before further identification studies are undertaken (Lodder, 1970).

India Ink Preparation to Detect Capsules. This preparation aids in the detection of capsules, a significant taxonomic character.

a. Place a loopful of India ink (Sanford) on a clean slide and a loopful of sterile distilled water close to the loopful of India ink.
b. Make a light emulsion of the yeast in the drop of water.
c. Mount with a cover slip such that the edge of the cover slip is placed at the outer edge of the drop of India ink and allowed to fall across both the ink and the yeast suspension, taking care to avoid air bubbles.
d. Immediately examine the area of the slide where the ink and water suspension meet. Capsules are seen easily in this area.

Characterization of Vegetative Growth on Corn Meal Tween Agar (Luecke Plate). The type of vegetative growth can be detected by growing the yeast isolates on corn meal tween agar, or Luecke plates. The Luecke plate is a combination of Wickerham's (1951) description of the Dalmau plate (Difco, 1972b), and our modification of Haley's technique (1971).

A corn meal tween plate (CMT) (Difco, 1972a; Walker and Huppert, 1960) is divided into four sections (Figure 1). Two sections are used for unknowns, the third and fourth for the two controls. A pure colony of yeast is touched with the tip of a sterile inoculation needle and a single streak is made on the CMT plate from near the edge of the petri dish down the center of the section of agar. The streak barely scarifies the medium (streak 1 in Figure 1). Starting from the edge of the petri dish a sterile applicator stick streaks back and forth along streak 1 (streak 2 in Figure 1). Although the span of streak 2 may be greater near the edge of the petri dish, note that it becomes narrow toward the center. A sterile square cover slip is placed over streaks 1 and 2 so that the streaks and a portion of the unstreaked medium are completely covered by the cover slip. This is an important and necessary step since observation of growth ultimately takes place at the edge of streak 2 (at the junction of the streaked and unstreaked medium under the cover slip). It is here that typical yeast morphology is seen and purity ascertained. The plate is incubated at 25°C for 48-72 hours in a moist chamber and then examined for typical blastospores and/or hyphae and/or arthrospores.

A control plate with *C. albicans* and one with a *Trichosporon* sp. are also streaked to compare with each plate containing unknowns.

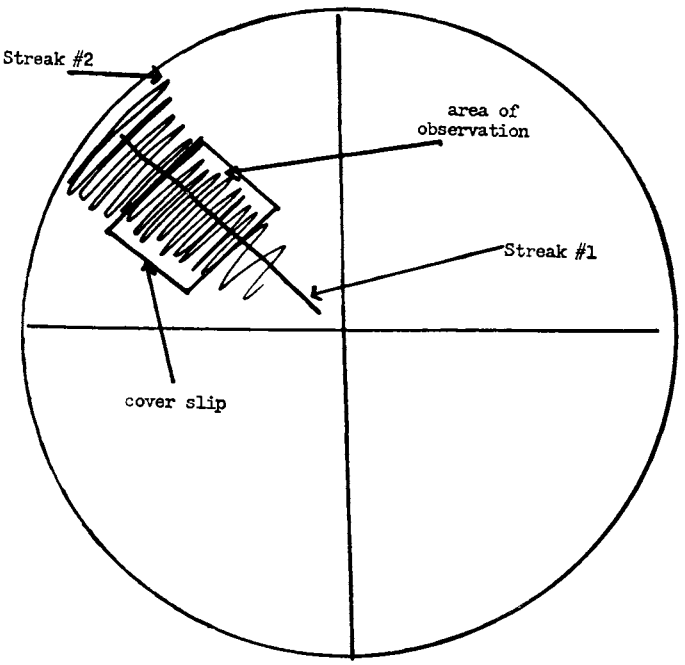

Figure 1. Method of streaking a Luecke plate.

Aberrant Yeasts Identified. Normal morphology of aberrant yeasts can be induced in the laboratory by prolonged incubation of aberrant yeasts in the Luecke plate and/or yeast malt agar for up to 10 days at room temperature in a moist chamber. The outermost isolated colonies on both media reveal the typical yeast structures not seen after only one or two days.

Assimilation and Fermentation Tests. Preparation of inoculum A. Two successive transfers of the yeast to yeast malt agar (YM) are ideal before testing for all physiological characteristics (Lodder, 1970). After the second transfer to YM agar, the inoculum is prepared by suspending a small amount of yeast from YM agar at the end of a sterile bacteriological loop in 2-5 ml of sterile 0.85% NaCl. This suspension is washed two times with sterile saline; 0.1 ml of this washed suspension is added to 4.5 ml of sterile 0.85% NaCl to eliminate the nitrogen and carbohydrate carryover from the original medium, a possibility which could interfere with studies of nitrogen and carbon assimilation and carbohydrate fermentation (Lodder, 1970).

Carbohydrate Assimilation Tests. Bacto yeast nitrogen base (YNB) as described by Dolan (1971) is used in this study. The technique includes the addition of sugar impregnated discs (Bacto differentiation discs) to a YNB agar surface previously inoculated with the washed yeast suspension. The YNB is dissolved in distilled water (11.7 gm:10 ml distilled water) and passed through a Swinnex-47 millipore filter (0.45μ pore diameter). Of this solution 88 ml are added to 1000 ml of a 2% sterile agar solution. Fifteen-ml portions are dispensed into plastic petri dishes. Inoculum A is used to flood the plate, and the excess drained off. To each plate are added four sugar impregnated discs (Bacto differentiation discs), and the plate is incubated at 30°C for 24-48 hours.

Growth immediately surrounding the discs indicates assimilation of the sugar by the yeast; clear areas around the discs indicate a lack of assimilation.

Positive controls: *Candida guilliermondii* for assimilation of all sugars, except lactose; *C. pseudotropicalis* for assimilation of lactose.

Negative controls: *Candida krusei.*

Fermentation Tests. These studies are performed according to Wickerham (1951). The medium contains 0.45% yeast extract, 0.75% peptone, and approximately 1 mg bromthymol blue sufficient to produce a green color in the medium. The yeast extract-peptone broth is dispensed in 2 ml quantities in 1½ x 150 mm screw-capped tubes. Durham tubes (50 x 6 mm) are added and the medium is autoclaved 15 minutes at 15 p.s.i. Sugars are prepared individually as 6% aqueous solutions, except raffinose which is prepared as a 12% solution. Each is sterilized with a Swinnex-47 millipore filter and stored until needed. One ml quantities are added to 2 ml of yeast-peptone broth.

The fermentation broth is inoculated with 0.1 ml of inoculum A. Tubes are shaken and incubated at room temperature or at 34-37°C, and examined regularly for 10-14 days.

Durham tubes are used to detect CO_2 production resulting from fermentative action. A pH change simply indicates the assimilation of a carbon compound.

To detect false negatives due to supersaturation of the broth, all tubes giving an acid reaction are shaken slightly and the cap vented. A rapid release of gas frequently follows.

Carbohydrates most helpful in identification include the following:

Hexose sugars—glucose (GLU), galactose (GAL);

Disaccharides—sucrose (USC), maltose (MAL), lactose (LAC), melibiose (MEL*), trehalose (TRE), cellobiose;

Trisaccharides—raffinose (RAF); and

Polysaccharides—inulin (INU).

The above 10 carbohydrates are used since keys to the majority of the species of the various genera are based on the fermentation and assimilation of these sugars.

Two *Candida* species, *C. tropicalis* and *C. pseudotropicalis*, may be necessary to run as positive controls with unknown yeasts; *Cryptococcus* species may serve as negative controls. Although each species ferments only several carbohydrates, the two together will provide fermentation of all the listed carbohydrates.

Nitrate Assimilation Test. The medium to test nitrate assimilation is prepared as follows. To Bacto yeast carbon base (11.7 gm) is added 100 ml distilled water. YCB is sterilized via the Swinnex-47 millipore filter and stored in the refrigerator until needed. The final medium, prepared by mixing 100 ml of sterile YCB with 900 ml of melted 2.25% sterile agar, is poured into petri dishes while still warm but not hot to touch after 0.1 ml of inoculum A is added to the petri dish. The mixture of warm YCB and inoculum A is swirled gently to mix, and allowed to cool.

One disc containing 1.0% KNO_3 is placed in the center of the inoculated YCB prepared above. The discs are prepared by soaking overnight in a petri dish containing approximately 20 ml of 1.0% KNO_3, and by decanting completely the excess nitrate, after which, the next day, the discs and the petri dish are autoclaved and dried. The petri dish is sealed with masking tape to avoid dehydration during storage at room temperature. The YCB plate with disc is incubated at 25°C. After 24 to 48 hours, growth around the disc where KNO_3 has diffused is a positive assimilation test.

Controls: positive control—*Cryptococcus albidus;* negative control—*Torulopsis glabrata* or *Candida albicans.*

Starch Production. The test for formation of starch has been employed primarily to separate the genus *Torulopsis* from *Cryptococcus* (Lodder, 1970). The ability to produce starch is often associated with the utilization of inositol and the ability to hydrolyze urea.

The YNBs containing various sugar discs are used to test for starch production. After an incubation period of 14 days, one drop of Lugol's iodine solution is added to the area around the glucose disc. Strains producing starch will turn the area blue to purple to green depending upon the intensity of starch.

A positive and a negative control are necessary. Starch results may be confusing unless both controls are used. The negative control may reveal a small black granule at the edge of the glucose disc, but this does not indicate positivity. A black ring around the disc with a diffusing black-green halo indicates starch production.

Positive control—*Cryptococcus* sp.; negative control—*Torulopsis* sp.

Medium for Ascospore Production. Two media are used to test for ascospore production. Gorodkowa's medium (Lodder, 1970) consists of 0.1% glucose, 1% peptone, 0.5% sodium chloride, and 2% agar. The medium is steamed to dissolve the agar and dispensed in 90 ml aliquots, autoclaved at 15 p.s.i. for 15 minutes, and poured into petri dishes as needed. Cultures are incubated at room temperature and observed for 4-6 weeks for ascospores.

Bacto malt agar (Lodder, 1970) is inoculated concomitantly with Gorodkowa's medium for ascospore production. (Malt agar also serves as a good medium for pigment production.)

Urease Production. Urea hydrolysis is detected by inoculation of Christensen's urea agar slants (Dolan, 1971) with one loopful of yeast and incubation at 30°C for 72 hours. In the presence of urease, the color of the medium changes to pink or red.

Positive control: *Rhodotorula* sp. or *Cryptococcus* sp.

Yeast Identification Guide. A guide for yeast identification is presented (Figure 2). Important steps in methodology are included. Spaces are provided for additional carbohydrates if more are needed to conclusively identify yeasts to species. When unknowns are identified, these guides may be filed alphabetically according to patient's name for future reference.

RESULTS AND DISCUSSION

Table 1 lists the genera and species of 3,474 yeasts isolated during a three-year study, the number and percent of each species isolated, and the various types of clinical material from which the species were isolated. Rare yeasts are indicated by few collections. Three sexual forms are represented, including *Pichia, Kluyveromyces,* and *Sporobolomyces;* the latter may be a Basidiomycete (Lodder, 1970). In

* Use in fermentation tests only when raffinose is fermented.

Name of patient ..... Identification No. .....
Date of specimen ..... Specimen ..... Ward .....
Initial isolate
1. Microscopic: Mixed ..... Pure .....
2. India ink for capsules: Yes ..... No .....
Pure culture
3. *Luecke plate* for vegetative growth characteristics.
   Blastospores only .....
   Pseudohyphae .....
   True hyphae .....
   Arthrospores .....
   Aberrant .....
   Control$_1$ = *C. albicans*
   Control$_2$ = *Trichosporon* sp.
4. Malt agar for pigmentation and ascospores.
   Pigment ..... Ascospores .....
   Pigment control = *Rhodotorula* sp.
   Ascospore control = *Saccharomyces* sp.
5. *Gorodkowa agar* for ascospores. Incubate at 25°C for 3-6 weeks.
   Ascospores .....
   Control = *Saccharomyces* sp.
6. *Growth* at 37°C ....., 45-48°C ....., 25°C .....
7. *Starch production*
   Unknown .....
   Positive control = *Cryptococcus* sp.
   Negative control = *Torulopsis* sp.
8. *Production of urease* (48 hrs. at 30°C).
   Unknown .....
   Positive control = *Cryptococcus* sp.
   Negative control = *Torulopsis* sp.
9. *Assimilation of NO_3* (48-96 hrs. at room temperature).
   Unknown .....
   Positive control = *Cryptococcus albidus* var. albidus.
   Negative control = *C. albicans* or *Torulopsis glabrata.*
10. *Assimilation of carbohydrates* (examine at 24 and 48 hrs., incubate at 30°C).

	glu*	gal	suc	mal	lac	mel	raf	inu	tre	
Control 1 (positive)	+	+	+	−	+	−	+	+	−	*C. pseudotropicalis*
Control 2 (positive)	+	+	+	+	−	+	+	+	+	*C. guilliermondii*
Control 3 (negative)	+	−	−	−	−	−	−	−	−	*C. krusei*
Unknown										

11. *Fermentation of carbohydrates* (examine at 1, 2, 3, 4, 7, 10 day; incubate at 37°C).

Control 1 (positive)	+	+	+	+	−	+ *C. tropicalis*
Control 2 (positive)	+	+	+	−	+	− *C. pesudotropicalis*
Control 3 (negative)	−	−	−	−	−	− *Cryptococcus* sp.

Unknown
Identification .....
Date .....

*glu = glucose
gal = galactose
suc = sucrose
mal = maltose
lac = lactose
mel = melibiose
ino = inositol
raf = raffinose
inu = inulin
tre = trehalose
+ = Acid and gas.

Figure 2. A guide for yeast identification.

TABLE 1. YEASTS ISOLATED DURING A THREE-YEAR PERIOD.

Organism	*Number of Strains*	*Percent of Total*	*Source*
Candida albicans	2,136	62.0	Sputum, lung biopsy, autopsy, wounds, pleural fluid, stool, ulcer, genitals, urine, blood
Candida tropicalis	627	18.0	Peritoneal fluid, sputum, wounds, blood, urine, bronchial washings
Torulopsis glabrata	111	3.2	Sputum, blood, urine, lung biopsy, bronchial washings, catheter
Candida stellatoidea	94	2.7	Sputum, blood, catheter, wounds
Geotrichum candidum	60	1.7	Stool, trachea, pleural fluid, lung abscess, lung biopsy, urine
Candida pseudotropicalis	40	1.2	Sputum, trachea, urine, blood, peritoneal fluid, endocarditis, ulcer, bronchial washings
Candida lipolytica	33	0.9	Throat swabs, wound, foot, catheter, autopsy
Candida krusei	28	0.8	Sputum, skin, urine, blood, trachea
Trichosporon fermentans	27	0.7	Sputum, bronchial washings, catheter
Candida rugosa	26	0.7	Sputum, bronchial washings, catheter, urine
Candida parapsilosis	25	0.7	Fistula, wounds, sputum
Trichosporon pululans	25	0.7	Urine, sputum, bronchial washings
Trichosporon capitatum	24	0.7	Urine, wounds, trachea, gastric washings
Candida guilliermondii	22	0.6	Lung biopsy, gastric washings, sputum
Trichosporon cutaneum	22	0.6	Urine, sputum, abdominal fluid, throat swabs, gastric washings
Torulopsis holmii	21	0.6	Foot, bronchial washings, catheters, wounds
Candida brumptii	19	0.6	Conjunctiva, catheter, skin (burn), urine
Trichosporon inkin	16	0.5	Sputum, throat swabs, skin lesion
Rhodotorula rubra	14	0.4	Leg ulcer, pleural fluid, scrotal tumor, sputum, urine
Torulopsis candida	14	0.4	Foot lesion, sputum, urine
Cryptococcus neoformans	13	0.4	CSF, skin
Geotrichum versiforme	13	0.4	Stool, sputum, bronchial washings
Sporobolomyces roseus	7	0.2	Sputum, lung biopsy, trachea
Trichosporon variabile	7	0.2	Sputum, skin lesion, ear
Saccharomyces cerevisiae	7	0.2	Sputum, wounds, bronchial washings
Torulopsis sphaerica	7	0.2	Sputum
Candida ater	7	0.2	Sputum, urine, skin
Saccharomyces telluris	7	0.2	Sputum, urine
Cryptococcus albidus	7	0.2	Lung biopsy, urine

Pichia guilliermondii	6	0.17	Sputum, skin, bronchial washings
Cryptococcus diffluens	5	0.5	Lung biopsy, urine
Kluyveromyces fragilis	2	0.05	Pharyngeal swab, sputum
Cryptococcus luteolus	2	0.05	Sputum, urine
Total	3,474		

addition to these genera, Lodder (1970) cites *Hansenula* and *Debaryomyces* as genera having medical importance. Their fermentation and assimilation characteristics are listed in Lodder (1970) and serve to assist in speciation of the various sexual forms. One area of difficulty in speciation of the various sexual stages may lie in the recognition of ascospores.

Description of Sexual Stages

Taxonomically, of the perfect genera *Saccharomyces, Kluyveromyces, Lodderomyces, Pichia, Hansenula,* and *Debaryomyces,* Lodder (1970) considers *Debaryomyes* the most primitive. From this genus probably arose the other five genera.

Two species of *Debaryomyces, D. marama* and *D. hansenii,* are the sexual stages of *Torulopsis candida.* Members of the genus *Debaryomyces* are small yeasts containing lipid globules which reproduce by multiple budding on CMT. Pseudomycelia are slight or absent. After two days in malt agar, cells are spherical to short-oval, single, in pairs or short chains. A one-month-old culture appears greyish white to yellowish.

Asci are formed by conjugation of the mother cell and the bud. The warts which develop on the resulting ascospore (produced on Gorodkowa's agar) are difficult to see under the light microscope, but can be seen well under oil or with phase microscopy. Usually one (rarely two) ascospore is produced in the ascus. Members of this genus ferment weakly or not at all. *D. marama* produces oval ascospores, 1-4 per ascus, while *D. hansenii* contains 1-2 spherical ascospores in its asci. Both species of *Debaryomyces* are perfect forms of *Torulopsis.*

Saccharomyces, a strong fermenter, reproduces asexually solely by multilateral budding. Blastospores are spheroidal, ellipsoidal, cylindrical, or elongate. True mycelia are absent but pseudohyphae may form. Asci, however, contain 1-4 spheroidal ascospores. Asci do not rupture at maturity. *Torulopsis bovina* is the imperfect form of *S. telluris.*

Hansenula, a second genus thought to have arisen from *Debaryomyces,* produces blastospores which bud on a narrow base at various places on the spherical, ellipsoidal, oblong, cylindrical, or elongated cells. At times these cells bear long tapers at one or both ends which are markedly elongated to thread-like. Primitive pseudohyphae are found on CMT under the cover slip and consist of cylindroidal cells which are highly branched and produce a few chains of cells (Blastospores). True hyphae are not produced on CMT.

Asci of *Hansenula* sp. have the shape of vegetative blastospores within which are produced 1-4 smooth walled ascospores which are hat-shaped, hemispherical, spherical, or Saturn-shaped. Ascospores are liberated upon maturity of the ascus.

H. jadenii is the only species of medical importance. Its ascospores are hat-shaped with a prominent brim. This species produces very few ascospores and may be the perfect stage of *C. utilis,* which is the only medically important species of *Candida* which assimilates nitrate. Assimilation and fermentation characteristics of *Hansenula* sp. can be found in Lodder (1970).

The genus *Pichia* produces blastospores of various shapes which reproduce by multilateral budding and, in most species, by pseudohyphae. True hyphae may be very limited.

Ascospores are spherical, hat- or Saturn-shaped, usually contain an oil droplet, are generally smooth, and asci contain one to four ascospores. *P. membranaefaciens* is the perfect form of *Candida valida* and produces ascospores which are spherical or hemispherical with or without a ledge or brim. One to four ascospores are produced per ascus. *P. guilliermondii* produces only hat-shaped ascospores.

Short descriptions of the ascosporic stages of *Hansenula* and *Pichia* have been presented. Lodder (1970) indicates that "even very primitive species of *Hansenula* and *Pichia* may produce diploid colonies; these may be distinguished from haploid colonies by differences in color, size and texture." Asci arise from diploid colonies. Different colonies on ascospore media, after two attempts at colony purification, may be a clue that asci are present. If different colonies are revealed on ascospore media and if the yeast produces hyphae, hyphal cuttings are suggested. The hyphal tips are dug out of the medium, squashed, and streaked on malt agar for isolated colonies. The resulting colonies are observed for asci and, if found, diploid cultures are seen at the edge of the colony.

If the yeast does not produce hyphae, sectors of a colony of different textures and colors are streaked on malt agar to isolate colonies, which then can be examined for asci.

Kluyveromyces produces blastospores which reproduce by budding. Pseudohyphae may be formed. Cells are spheroidal, ellipsoidal, cylindrical, or elongate. True mycelia are absent.

Asci rupture upon maturation and liberate ascospores which tend to agglutinate. Ascospores are crescentic, reniform, oblong with obtuse ends, spheroidal, or prolate-ellipsoidal. One to numerous smooth-walled ascospores are formed within an ascus.

Kluyveromyces is the perfect stage of *Candida macedoniensis, C. pseudotropicalis,* and *Torulopsis sphaerica.*

The blastospores of *Lodderomyces* are spheroidal, or ellipsoidal to cylindrical, and reproduce by budding. Pseudohyphae are abundant; they may be branched or the blastospores may be arranged in clusters or in branching clusters along pseudohyphal cells.

Mature ascospores are large, oblong, with obtuse to prolate ellipsoidal ends. They are occasionally slightly curved and not liberated from the ascus. One or rarely two ascospores are produced in each ascus. *L. elongisporus* is the only species in the genus and is considered to be the sexual phase of *Candida parapsilosis.*

Sporobolomyces reproduces vegetatively by simple budding and at times by the formation of pseudo- and/or true mycelia with crosswalls. Blastospores are ovoid to elongate, and on some of the vegetative cells ballistospores develop. Ballistospores are typically asymmetrical, kidney- or sickle-shaped, and develop in an oblique position at the tips of aerial sterigmata. Upon maturity, these spores are forcefully discharged into the air by a "droplet mechanism." In this process, a droplet of liquid is excreted at the tip of the sterigma near the hilum of the spore, and when the volume of the drop increases, the drop is discharged, carrying the ballistospore with it.

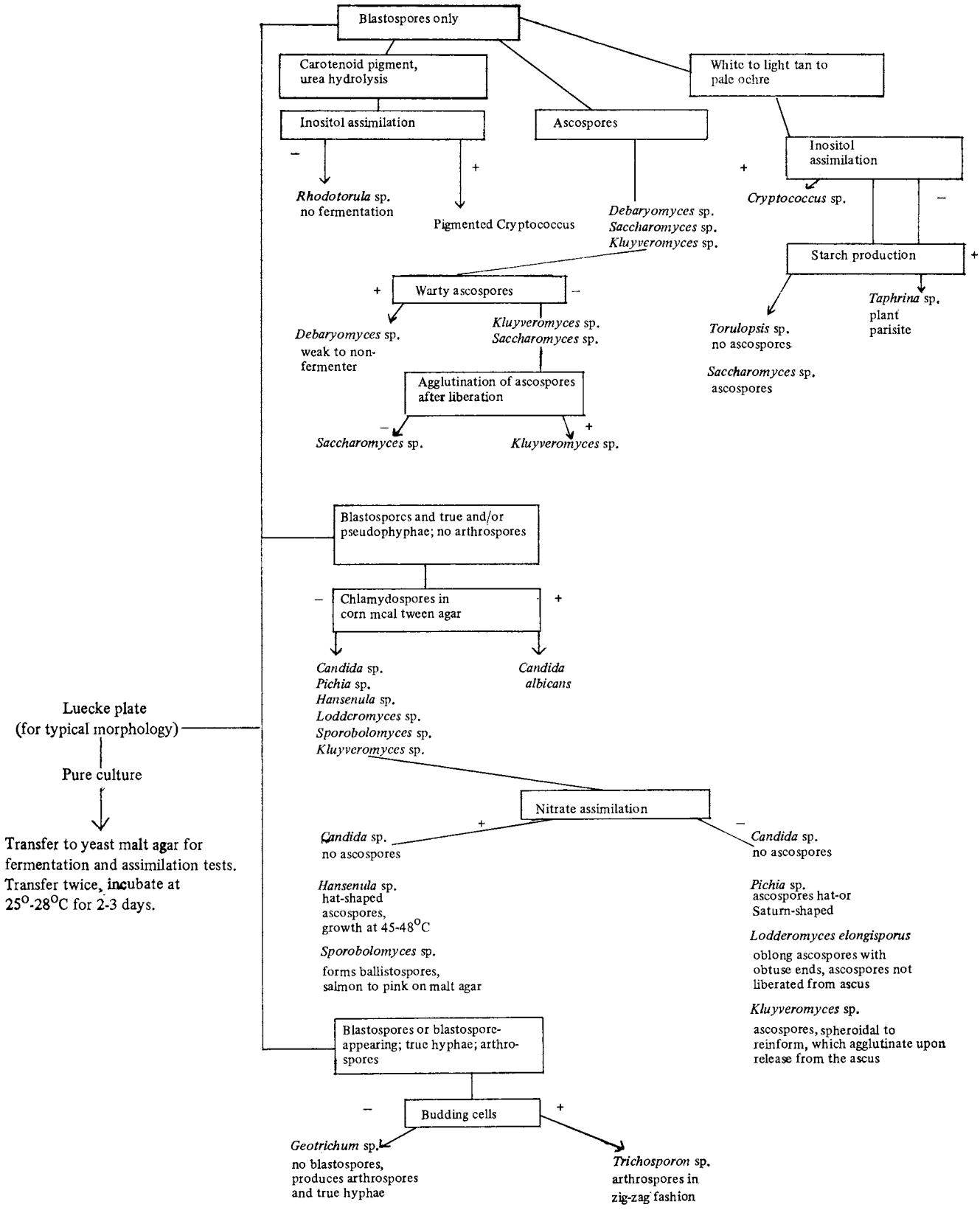

Figure 3. Scheme for the identification of yeasts from clinical material.

A red or salmon-pink color is produced by two species of medical importance on malt agar, but some species are not pigmented.

The cells forming ballistospores are considered by some authors to be reduced basidia (Lodder, 1970). Although cytological data has still not clearly demonstrated a sexual stage, many authors tend to link this genus with the Basidiomycetes.

Basidiomycete sexuality has also been suggested for *Candida albicans* and *C. stellatoidea*. The chlamydospores are considered "teliospore homologs" (Lodder, 1970). Telio- or chlamydospores are markedly influenced by environmental conditions and are stimulated by nutritionally deficient media (corn meal tween). The sexual stages of *C. albicans* and *C. stellatoidea* have been classified in a separate genus, *Syringospora* (Lodder, 1970).

Identical physiological characteristics often serve to connect a sexual genus with an asexual one. There are, however, sexual stages which have not yet been associated with an imperfect form and imperfect forms not yet associated with sexual forms. The Identification Scheme (Figure 3) attempts to assist in the identification of either stage. For this reason a yeast may be found in two areas of the Scheme.

Discussion of the Yeast Identification Guide (Figure 2). The methods necessary for accuracy in yeast identification have been presented. Their importance justifies the preparation of this Guide, which is in the form of a daily work sheet on which the result of each test is reported. The mere presence of each step on the chart reminds the mycologist what test is completed and what is yet to be performed.

The negative and positive controls are important steps in the chart, for unless the mycologists are certain the systems for testing unknowns are performing adequately, the accurate identification of the unknown is not assured. Only 10 stock cultures of knowns are needed for adequate controls for all steps in the Guide. They should be maintained in stock on YM agar. The knowns used in this laboratory are indicated next to each test in the guide.

Identification Scheme (Figure 3). A work sheet (Guide) replete with results probably contains all the information necessary for identification of the unknown. With the use of the Identification Scheme it is possible to identify all the yeast genera isolated during our three-year study as well as those yeasts cited by Lodder (1970) and discussed earlier which have medical importance.

Utilization of the Scheme begins with the microscopic appearance of the pure unknown on the Luecke plate (corn meal tween agar) (Walker, 1960). The unknown will respond in one of three ways: (1) produce blastospores only; (2) produce blastospores, true and/or pseudohyphae, but not arthrospores; or (3) produce blastospores (or the appearance of blastospores), true hyphae, and arthrospores. Identity of the yeast genus, and sometimes species, follows determination of the response of the yeast on the Luecke plate.

Production of Blastospores Only. If an unknown reveals blastospores without true or pseudohyphae, the unknown may belong to the genus *Rhodotorula, Cryptococcus, Saccharomyces, Torulopsis,* or *Debaryomyces*. Since *Kluyveromyces, Saccharomyces,* and *Debaryomyces* are Ascomycetes, an obvious second step in identification necessitates inoculation of Gorodkowa agar and malt agar for production of ascospores. (Ascosporic yeasts will be discussed later.)

Pigment production, or the lack of it, on malt agar and the presence or absence of urease will assist in identification. *Rhodotorula* or *Cryptococcus* may be pigmented and may hydrolyze urea (Lodder, 1970), an ability usually lacking in ascospore producers.

On the basis of inositol assimilation results, the unknown may be identified as a *Rhodotorula* or *Cryptococcus* sp. (*Cryptococcus* assimilates inositol while *Rhodotorula* does not.) Starch production results will further assist in identification since *Cryptococcus* sp. produce starch while most *Rhodotorula* do not. Speciation is possible after assimilation testing is completed.

Most cryptococci are tan to white in color. Such an unknown with encapsulated blastospores is considered a *Cryptococcus* sp. if it assimilates inositol and produces starch. Note that a pigmented *Cryptococcus* exists and such a species could erroneously be placed in the genus *Rhodotorula.*

Ten species of *Cryptococcus* have been isolated from clinical material (Lodder, 1970).

Taphrina may also produce ascospores. This genus includes well-known plant pathogens and grows saprophytically. But *Taphrina* has been misidentified in the past as a *Rhodotorula* and/or *Cryptococcus* due to its resemblance to these two genera. For this reason it is included here. *Taphrina* is yellow-tan (pale ochre) in color, neither ferments sugars nor assimilates nitrate, but does produce starch (Lodder, 1970). *Saccharomyces,* although an Ascomycete, does not produce starch. This test serves to differentiate *Saccharomyces* from *Taphrina.* An unknown is identified as a species of Saccharomyces if it produces blastospores and typical ascospores, neither assimilates inositol nor hydrolyzes urea, and fails to produce starch. Four species of *Saccharomyces* have been isolated from disease processes (Lodder, 1970). Two of these have been isolated in this laboratory and are seen in Table 1. Fermentation and assimilation characteristics of all medically important species are seen in Lodder (1970).

If the unknown yeast is a *Torulopsis,* it is differentiated from *Saccharomyces* by its lack of ascospore production. Fermentation and assimilation characteristics of all species of *Torulopsis* are found in Lodder (1970).

The perfect stage of *T. candida* is *Debaryomyces marama* and *D. hansenii.* Of interest is that both species of *Debaryomyces* have been isolated from clinical specimens. *D. hansenii* has already been discussed in the introduction.

An ascospore-producing unknown colony of yeast on malt agar which does not produce capsules, starch, or fit the characteristics of *Rhodotorula, Torulopsis,* or *Cryptococcus,* is likely to be a species of *Debaryomyces, Kluyveromyces,* or *Saccharomyces.* These genera are differentiated from each other by the warty ascospores of *Debaryomyces* which are better seen under oil, the inability of this genus to ferment, the smooth spherical ascospores of *Saccharomyces,* and the smooth reniform to spheroidal ascospores of *Kluyveromyces.* The latter two genera ferment vigorously.

Three species of *Kluyveromyces* (*K. marxianus, K. fragilis,* and *K. lactis*) have been isolated from clinical material. A representative of this genus has been found in clinical specimens in this laboratory (Table 1). Of interest is that *K. marxianus* is the perfect form of a species of *Candida* also implicated in disease (*C. macedoniensis*). *K. fragilis* is the perfect form of *C. pseudotropicalis,* while *K. lactis* is the ascosporic stage of *Torulopsis sphaerica.*

Debaryomyces, an ascosporic genus, produces only blastospores which are small and contain lipid globules. This genus has been isolated from a patient with angina, from interdigital mycosis and diseased nails, from the throat and sputum,

skin scales, and foods (Lodder, 1970). Its most outstanding characteristic is its warty ascospores (not always seen with a light microscope) and it is this characteristic which separates this genus from *Kluyveromyces;* the latter forms smooth ascospores which are spheroidal to reniform and agglutinate upon liberation from the ascus.

If the unknown belongs to one of the genera which do not assimilate nitrate (*Pichia, Candida* sp., *Kluyveromyces,* and *Lodderomyces*), it may be a *Candida* species, none of which produces ascospores, which serves to separate *Candida* from the other genera in this group. *Pichia, Lodderomyces,* and *Kluyveromyces* produce characteristic ascospores which have been described in the section which discusses sexual stages. Of interest is that *P. guilliermondii* is the perfect stage of *Candida guilliermondii;* therefore their biochemical characteristics are identical. Assimilation and fermentation characteristics of this genus are found in Lodder (1970).

Only one species of *Lodderomyces, L. elongisporus,* exists; it is identified by its oblong ascospores (Lodder, 1970) which are not liberated from the ascus.

The vegetative characteristics of *Candida* species are well exhibited on the Luecke plate. This medium is used as presumptive evidence that an unknown belongs to this genus (Haley, 1971), and often assists in the choice of carbohydrates needed for speciation. Their fermentation and assimilation characteristics are seen in Lodder (1970).

Kluyveromyces is the genus in which the sexual forms of two species of *Candida* and a species of *Torulopsis* are classified. *Candida* produces true or pseudohyphae, but *Torulopsis* does not. Because these asexual forms are morphologically distinct, the genus *Kluyveromyces* is found in two areas in the Identification Scheme—in "blastospores only" and in "blastospores with true and/or pseudohyphae."

Assimilation and fermentation test results of the three ascosporic genera are found in Lodder (1970).

Production of Blastospores, True or Pseudohyphae, But Not Arthrospores. If the unknown isolate on a Luecke plate reveals blastospores, true or pseudohyphae, and no arthrospores, the Identification Scheme suggests that the unknown may be, among other genera, a *Cryptococcus,* since some strains produce pseudohyphae. The unknown may be a *Saccharomyces* since some strains produce poorly developed pseudohyphae. To rule out or to consider one of these as the unknown genus, follow the scheme from "white to light tan to pale ochre."

When the above two genera are ruled out there are other genera to which the unknown may belong and which produce true and/or pseudohyphae plus blastospores. These include *Candida, Pichia, Hansenula, Kluyveromyces, Sporobolomyces,* and *Lodderomyces.*

Of the many medically important species of *Candida* and other yeast genera, *C. albicans* is most often found in clinical specimens, and usually in sputum. The identification of *C. albicans* from a clinical specimen containing mixed flora, particularly where *C. albicans* is considered to be a part of the normal flora, has no diagnostic value. *C. albicans* must be purified and the amount in the original isolate estimated. Large numbers of *C. albicans* colonies in several sputum specimens do have diagnostic value. Therefore purification procedures and steps which ascertain purity are necessary.

After the purification procedure (outlined in this report under Methods) the Luecke plate is inoculated to ensure culture purity. Corn meal tween agar is well-known as one which stimulates typical morphology of all yeasts including the typical chlamydospores of *C. albicans* (Walker, 1960; Lodder, 1970). The Luecke plate therefore assures purity, and contains a "built-in" mechanism for stimulating all yeasts to reveal typical morphology, including the large number of typical chlamydospores of *C. albicans.*

Rarely do we depend on one technique or one step for the identification of any species. Additional steps are often required for the identification of atypical strains. For example, absolute dependency on corn meal tween for the identifications may be deceptive since *C. tropicalis* and *C. stellatoidea* have been known to produce chlamydospores on this medium. Furthermore, a rare strain of *C. albicans* may not produce chlamydospores (Walker and Huppert, 1959). However, morphologic characteristics of *C. stellatoidea* and *C. tropicalis* are often clues that *C. albicans* is not present on corn meal tween, notwithstanding the occasional chlamydospores which may be seen. When formed, the chlamydospores of *C. tropicalis* are scanty and not typical of those of *C. albicans.*

If a yeast fails to reveal identifiable structures on corn meal tween in 24 hours, the culture is allowed additional incubation time, and assimilation and fermentation tests are performed immediately. Additional incubation time on corn meal tween has ultimately revealed typical morphology which was shown by fermentation and assimilation tests.

A well-known procedure for *C. albicans* identification is the "germ tube test" (Blair et al., 1970). Identification is effected in a few hours by the production of "germ tubes" which are filamentous outgrowths from the blastospores or chlamydospores after exposure to mouse peritoneal fluid *in vivo* or to human or animal serum *in vitro.* This technique may be helpful for rapid identification of atypical *C. albicans* strains since atypical cultures tend to prolong the identification period. However, additional studies should be undertaken to determine whether these atypical strains would produce typical germ tubes. This technique would also be helpful in ruling out the presence of *C. albicans* in the event a *C. tropicalis* or *C. stellatoidea* produced chlamydospores on corn meal tween. However, our identification steps include assimilation and fermentation studies on all atypical *Candida* isolates and all species of *Candida* which do not produce the typical morphological characteristics of *C. albicans* on corn meal tween. *C. stellatoidea* and *C. tropicalis* would be identified using these techniques, and therefore additional methods are not necessary.

To summarize, we prefer the use of corn meal tween agar to culture purity and to stimulate typical morphologic characteristics, including those of *C. albicans.* If *Candida* species other than *albicans* are present, or if atypical chlamydospore-producing yeasts are present, fermentation and assimilation tests are performed while the yeast undergoes prolonged incubation on corn meal tween. These steps serve to identify each isolate to species while the one-step germ tube technique merely ascertains the presence of *C. albicans.* Since our procedures not only identify *C. albicans* but other yeast genera to species a test which identifies only one species is not necessary.

When typical *C. albicans* chlamydospores and morphology are not produced on corn meal tween, sugar assimilation and fermentation tests are warranted. Only one medically important species of *Candida, C. utilis,* assimilates nitrate (Lodder, 1970). This is the only genus of this group (*Candida, Pichia, Hansenula, Lodderomyces, Sporobolomyces,* and *Kluyveromyces*) which does not produce ascospores or ballisto-

spores. If the unknown assimilates nitrate but is not *C. utilis*, other genera in this group must be considered. Assimilation and fermentation results will help to identify the *Candida* species (Lodder, 1970). The genera *Lodderomyces, Pichia,* and *Kluyveromyces*, and the majority of *Candida* species cannot assimilate nitrates. Ascospore media results are important in identification of those genera which may or may not assimilate nitrate. Of those genera which assimilate nitrate, *Hansenula* produces hat- or Saturn-shaped ascospores and grows well at 45-48°C. These characteristics differentiate *Hansenula, Candida*, and important species of *Sporobolomyces.*

Hansenula jadinii has been isolated from clinical material and is the perfect stage of *C. utilis*. Its fermentation and assimilation characteristics are seen in Lodder (1970).

Sporobolomyces is differentiated from *Candida* species in its production of characteristic ballistospores. The latter have been described under the description of sexual stages. Two species of *Sporobolomyces* have attained importance in medical mycology. Their fermentation and assimilation characteristics are listed in Lodder (1970).

Production of Blastospores, Blastospore-Like Cells, True Hyphae and Arthrospores. The presence of these characteristics on the Luecke plate indicates that the unknown belongs to one of two genera, *Geotrichum* or *Trichosporon. Geotrichum,* although often yeast-like in culture, is not classified as a yeast. Yet its blastospore-like arthrospores often seen in primary isolates are frequently interpreted as yeast cells. Growth of *Geotrichum* sp. on CMT for three days or more will reveal characteristic arthrospores. Depending on the species, a *Geotrichum* may reveal asymmetric germ tube formation. But this characteristic is often confused with the zigzag fashion of arthrospore production in *Trichosporon species. Trichosporon*, however, produces budding blastospores in addition to arthrospores, which serves to differentiate *Trichosporon* from *Geotrichum*, which does not form budding cells.

Those species of *Trichosporon* recovered from clinical specimens in this laboratory are listed in Table 1.

ACKNOWLEDGMENTS

The authors wish to thank Mrs. Edith McKown, Secretary, Laboratory Service, Wadsworth V.A. Hospital, for her diligence in the preparation of this manuscript.

REFERENCES CITED

DIFCO. 1972. Detection and identification of *Candida albicans.* Technical Information No. 0165. p. 1-12.

DIFCO. 1972. Media for the classification of yeasts. Technical Information No. 0224. p. 1-8.

DOLAN, C. T. 1971. A practical approach to identification of yeast-like organisms. *Amer. J. Clin. Path.* 55: 580-590.

HALEY, L. D. 1971. Identification of yeasts in clinical microbiology laboratories. *Amer. J. Med. Tech.* 37: 125-131.

LODDER, J. 1970. The yeasts: A taxonomic study. North-Holland Publishing Co., Amsterdam-London.

LOURIA, D. B., A. BLEVINS, D. ARMSTRONG, R. BURDICK, and P. LIEBERMAN. 1967. Fungemia caused by "nonpathogenic yeasts." *Arch. Intern. Med.* 119: 247-252.

PAINTER, B. G., and H. D. ISENBERG. 1973. Isolation of *Candida parapsilosis:* A report of 2 cases. *Amer. J. Clin. Path.* 59: 62-65.

SILVA-HUNTER, M. 1970. Yeasts. Pages 352-363 *in* J. E. Blair, E. H. Lennette, and J. P. Truant, eds. Manual of clinical microbiology. Am. Soc. Microbiol., Bethesda, Md.

WALKER, L. J., and M. HUPPERT. 1959. A rapid, reliable technic for the identification of *Candida albicans. Amer. J. Clin. Path.* 31: 551-558.

WALKER, L. J., and M. HUPPERT. 1960. Cornmeal tween agar: An improved medium for the identification of *Candida albicans. Tech. Bull. Reg. of Med. Tech.* 33: 190-195.

WICKERHAM, L. J. 1951. Taxonomy of yeasts. U.S. Dept. of Agric. Tech. Bull. 1029. 56 pp.