A SIMPLE DISK-DIFFUSION TEST FOR DIFFERENTIATION OF YEAST SPECIES

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SUMMARY. A disk-diffusion method for identification of yeasts was developed that depended on their different but distinct susceptibilities to the following chemicals: janus green, ethidium bromide, 2, 3, 5-triphenyltetrazolium chloride, brilliant green, cycloheximide and rhodamine 6G. For 594 of 623 routinely isolated yeasts, the disk-diffusion and the commercial API 20C auxanogram tests gave the same identification, an agreement of 95.3%. Only 8 of 1052 isolates from clinical specimens were not identified by the disk-diffusion method. The method is simple, inexpensive and technically straightforward and for most isolates gives an identification in 24 h.

INTRODUCTION

Many different factors including intensive-care support and the use of corticosteroids, antibiotics, immunosuppressive drugs and oral contraceptives have increased the susceptibility of patients to infections by yeasts (Hart *et al.*, 1969; Meinhof, 1972; Cho and Choi, 1979; Seeliger, 1980). In order to predict appropriate antimycotic therapy and to monitor development of resistance and spread of infection, methods of differentiation that are rapid, reliable and easy are needed.

Conventional and commercial systems for the identification of yeasts are based on different properties that include colony morphology, microscopical appearance and fermentative and assimilative reactions. But these methods are labour-intensive, time consuming and expensive and alternative methods are worthy of investigation.

Yeasts are known to be sensitive to dyes (Sobczak, 1981), an observation affording a possible basis for differentiating species. Accordingly, the potential of a disk-diffusion test for the identification of yeasts was investigated.

MATERIALS AND METHODS

Yeast strains. The following reference strains were obtained from the Deutsche Sammlung von Mikroorganismen (DSM): *Candida albicans* strains DSM 70006 and 70014; *C. guilliermondi* strains DSM 70051, 70052 and 70057; *C. krusei* strain DSM 70075; *C. parapsilosis* strain DSM 70126; *C. tropicalis* strain DSM 70151; *Cryptococcus albidus* strain DSM 70403; *Cr. laurenti* strain DSM 70766; *Cr. neoformans* strain DS 70219; *Geotrichum candidum* strain DSM 1240; *Rhodotorula rubra* strains DSM 70403 and 70404; *Saccharomyces cerevisiae* strains DSM 70449

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and 70451; *Torulopsis candida* strain DSM 70590; *T. glabrata* strain DSM 70614. Other standard strains used were: *C. lipolytica* strains Hantschke 5306 and 5104 II; *C. pseudotropicalis* Gedek; *C. rugosa* strains Hantschke 1545b, 1625, 1812b and 1958c; *C. stellatoidea* strain Rieth; *T. candida* strains Hantschke 1586b II and 1772b II.

A further 1052 isolates in two series from clinical specimens were examined. Series A isolates (623) were identified by microscopic examination, formation of germ tubes and chlamydospores, resistance to cycloheximide, growth at 25° and 37°C and growth on blood agar, and also by means of the commercially available API 20C AUX kit (API/bioMérieux GmbH, Box 1204, D-7440 Nürtingen, FRG). Eighty-seven of these strains were also identified by conventional methods (Lodder and Kreger-van Rij, 1967). Series B isolates (429) were examined by the disk-diffusion method only.

Chemicals. Stock solutions of the following chemicals were prepared in distilled water and autoclaved at 121°C for 15 min unless otherwise stated: janus green (Merck, Box 4119, 6100 Darmstadt 1, FRG) in Sabouraud Liquid Medium (CM 147, Oxoid) 5 g/L, adjusted to pH 8.0 with 1N NaOH; ethidium bromide (Serva, Box 105260, D-6900 Heidelberg 1, FRG) 1.25 g/L; 2, 3, 5-triphenyltetrazolium chloride (Serva) 5 g/L, stored in the dark; brilliant green (Merck) 50 mg/L; cycloheximide (Upjohn, Box 449, 6148 Heppenheim, FRG) 500 mg/L in sterile distilled water; rhodamine 6G (Merck) 2 g/L in ethanol.

Preparation of disks. Sensi-Blanck-Disks, 6 mm in diameter (BBL, Becton Dickinson, Box 101629, D-6900 Heidelberg 1, FRG) were placed in flat-bottomed microtitration plates, and each disk was impregnated with stock solution (30 µl) and dried overnight at 37°C. Thus, disks

FIG. 1.—Disk-diffusion test with *C. albicans* (inoculated by swab rotation). Disks contain: (1) Janus green; (2) ethidium bromide; (3) 2, 3, 5-triphenyltetrazolium chloride; (4) brilliant green; (5) cycloheximide; (6) rhodamine 6G.

contained the following amounts of chemicals: janus green (JG) 150 µg, ethidium bromide (EB) 37.5 µg; 2,3,5-triphenyltetrazolium chloride (TTC) 150 µg; brilliant green (BG) 1.5 µg; cycloheximide (CY) 15 µg; and rhodamine 6G (R6G) 60 µg.

Inoculation. Part of a colony from a culture on Sabouraud Dextrose Agar (CM41, Oxoid) was suspended in 10 ml of sterile saline (NaCl, 0.9% w/v in water) to give a light opalescent suspension. Plates of Sabouraud Dextrose Agar were inoculated by rotating a swab, soaked briefly in the undiluted suspension, over the agar or by flooding the surface of the agar with 5 ml of a 1 in 5 dilution of the suspension, removing excess fluid after 10–30 s and leaving the plate at room temperature until the surface of the agar was dry.

Disk-diffusion test. Disks (1–6) were applied to the agar in a set pattern, as shown in fig. 1. Because there were large zones of inhibition around the disks containing cycloheximide with some strains, that disk was placed at position 5 (see fig. 1). After incubation at 25° or 37°C for 24 or 48 h, depending on the species, zones of inhibition of growth were measured (mm) and recorded. Special morphological features were also noted.

Strains were recorded as sensitive or resistant to the six chemicals in disks 1–6, and the resistance profile coded. Thus, for example, a strain of *C. albicans* of code 100406 was inhibited by chemicals 1, 4 and 6 (JG, BG and R6G, respectively) but resistant to chemicals 2, 3 and 5 (EB, TTC and CY, respectively); the numbers of the latter chemicals are replaced by 0 in the code (see table I).

RESULTS

In preliminary tests, more than 80 chemicals, many of them dyes, were tested over a range of different concentrations in disk-diffusion tests for their ability to inhibit and differentiate between reference strains of yeast. These studies suggested that six chemicals were of value for differentiation purposes. Thus, the reference strains were tested in disk-diffusion tests to observe their sensitivity or resistance to these six chemicals at appropriate concentrations, and the results of these studies are shown in table I. Apart from the *Cr. albidus* and *S. cerevisiae* strains, which had the same profile

TABLE I
Representative profiles of reference strains of yeasts

Species	Reaction* with chemical†						Code
	JG	EB	TTC	BG	CY	R6G	
<i>G. candidum</i>	—	—	—	—	—	—	000000
<i>C. rugosa</i>	—	—	—	+	+	—	000450
<i>Cr. laurenti</i>	—	+	—	—	+	—	020050
<i>C. lipolytica</i>	—	+	—	+	—	—	020400
<i>C. parapsilosis</i>	—	+	—	+	+	—	020450
<i>T. candida</i>	—	+	—	+	+	+	020456
<i>T. glabrata</i>	—	+	+	—	+	—	023050
<i>C. krusei</i>	—	+	+	+	+	+	023456
<i>C. albicans</i>	+	—	—	+	—	+	100406
<i>C. stellatoidea</i>	+	—	+	+	—	+	103406
<i>R. rubra</i>	+	+	—	+	—	+	120406
<i>Cr. neoformans</i>	+	+	—	+	+	—	120450
<i>C. guilliermondii</i>	+	+	—	+	+	+	120456
<i>C. pseudotropicalis</i>	+	+	+	+	—	+	123406
<i>S. cerevisiae</i>	+	+	+	+	+	—	123450
<i>Cr. albidus</i>	+	+	+	+	+	—	123450
<i>C. tropicalis</i>	+	+	+	+	+	+	123456

* + = Inhibition; — = no inhibition (For mean diameter, mm, of zones of inhibition, see table V).

† For abbreviations of chemicals, see *Materials and Methods*.

(code 123450) but which may be distinguished readily on the basis of colonial morphology, each species apparently possessed a typical profile.

Clinical isolates

The 623 clinical isolates in series A were examined by the disk-diffusion test and by the API 20C auxanogram; 615 (98.7%) were identified by the API 20C auxanogram and 617 (99.0%) by the disk-diffusion test. For 594 isolates, both methods gave the same identification, an agreement between them of 95.3%. Six isolates not identified by the disk-diffusion test were identified by the API 20C auxanogram as *Hansenula anomala* (1 strain), *Hanseniaspora ovarum* (1), *C. zeylanoides* (2) and *C. lambica* (2), species not listed among our reference series for the disk method. Eight isolates not identified by the API 20C auxanogram were identified by the disk-diffusion test as *C. stellatoidea* (4 strains), *C. krusei* (2), *C. tropicalis* (1) and *C. parapsilosis* (1). Fifteen isolates were identified as species within the test group by both methods but gave divergent results (table II). Conventional methods verified the identification from disk diffusion for five strains and by API 20C auxanogram for four isolates (table II); six strains were not tested by conventional methods.

The disk-diffusion results for the 594 clinical isolates with identifications in agreement by both methods are summarised in table III. For the species *C. tropicalis* (55 isolates), *C. guilliermondi* (12), *C. pseudotropicalis* (6), *C. lipolytica* (3), *C. stellatoidea* (3), *R. rubra* (2) and *T. candida* (4), all strains of each species gave the same code (table III), although it must be noted that only a few strains of some species were examined.

Within the other five species, two or more groups were recognised (table III). Of the 430 isolates identified by API 20C-testing as *C. albicans*, 418 gave the same disk-diffusion profile as the reference strains of *C. albicans*. Of the remainder, 10 isolates were slightly inhibited by ethidium bromide and two strains were not inhibited by janus green; their profiles deviated only slightly from that of typical strains. Furthermore, these strains with the less common profiles were germ-tube positive, therefore all three profiles probably indicated *C. albicans*. Characteristic for all strains of *C. albicans* were

TABLE II
Summary of divergent identifications by different methods

Disk diffusion	Identification (number of isolates) by API 20C auxanogram	Conventional methods*
<i>C. guilliermondi</i> (1)	<i>C. parapsilosis</i> (1)	Not identified (1)
<i>C. tropicalis</i> (3)	$\left\{ \begin{array}{l} C. parapsilosis (1) \\ C. guilliermondi (1) \\ R. pilimanae (1) \end{array} \right\}$	Not identified (3)
<i>C. pseudotropicalis</i> (3)	$\left\{ \begin{array}{l} C. albicans (1) \\ C. tropicalis (2) \end{array} \right\}$	$\left\{ \begin{array}{l} C. pseudotropicalis (2) \\ C. tropicalis (1) \end{array} \right\}$
<i>C. stellatoidea</i> (8)	<i>C. albicans</i> (8)	$\left\{ \begin{array}{l} \text{Not identified (2)} \\ C. albicans (3) \\ C. stellatoidea (3) \end{array} \right\}$

*See Lodder and Kreger-van Rij (1967).

TABLE III
The behaviour of 594 clinical isolates of yeasts in the disk-diffusion test

Species	Inhibition* with chemical†						Code	Number of isolates
	JG	EB	TTC	BG	CY	R6G		
<i>C. albicans</i>	{ +	-	-	+	-	+	100406	418
	{ +	+	-	+	-	+	120406	10
	{ -	-	-	+	-	+	000406	2
<i>C. guilliermondi</i>	{ +	+	-	+	+	+	120456	12
<i>C. krusei</i>	{ +	+	+	+	+	+	123456	8
	{ -	+	+	+	+	+	023456	3
<i>C. lipolytica</i>	{ -	+	-	+	-	-	020400	3
	{ +	+	-	+	+	-	120450	4
<i>C. parapsilosis</i>	{ -	-	-	+	+	-	000450	4
	{ -	+	-	+	+	-	020450	1
<i>C. pseudotropicalis</i>	{ +	+	+	+	-	+	123406	6
<i>C. stellatoidea</i>	{ +	-	+	+	-	+	103406	3
<i>C. tropicalis</i>	{ +	+	+	+	+	+	123456	55
<i>Geotrichum</i> sp.	{ -	-	-	-	-	-	000000	1
	{ -	+	-	+	-	-	020400	1
<i>R. rubra</i>	{ +	+	-	+	-	+	120406	2
<i>T. candida</i>	{ -	+	-	+	+	+	020456	4
	{ -	+	+	-	+	-	023050	29
	{ -	-	+	-	+	-	003050	7
<i>T. glabrata</i>	{ -	+	+	+	+	-	023450	7
	{ +	+	+	+	+	-	123450	7
	{ +	+	+	-	+	-	123050	5
	{ -	-	+	+	+	-	003450	2

* + Inhibition; - = no inhibition (for mean diameter, mm, of zones of inhibition, see table V).

† For abbreviations of chemicals, see *Materials and Methods*.

small- to medium-sized zones of inhibition by brilliant green and rhodamine 6G and, for most strains, a small zone of inhibition with janus green (fig. 1).

There were two groups of *C. krusei* strains, three isolates giving the same profile as the reference strains and the other eight isolates a resistance profile only slightly different from that of the reference strains. Regardless of the subgroup, strains of *C. krusei* showed within the zone of inhibition around ethidium bromide evidence of regrowth, and all isolates were very sensitive to cycloheximide (table III).

Among the nine isolates of *C. parapsilosis*, only one had the same profile as the reference strain but the other two profiles, represented by four isolates each, closely resembled that of the reference strain (table III).

Only two isolates of *Geotrichum* species were identified, one of which, like the reference strain, was not inhibited by any of the six chemicals; the other was inhibited by ethidium bromide and brilliant green.

The greatest variety of profiles was found with the 57 strains identified by API 20C auxanogram as *T. glabrata*, which usually showed characteristic regrowth within the zones given by triphenyltetrazolium chloride and ethidium bromide (fig. 2). Furthermore, strains of *T. glabrata* were resistant to rhodamine 6G (table III).

The 429 clinical isolates in series B were tested by the disk diffusion method only. Apart from two isolates, also not identified by the API 20C auxanogram method, all were readily identified. The results for series A and B are combined in table IV, showing

FIG. 2.—Disk-diffusion test with *T. glabrata* (inoculated by swab rotation). Characteristic regrowth within zones given by ethidium bromide (disk 2) and 2, 3, 5-triphenyltetrazolium chloride (disk 3). Disks applied in same order as in fig. 1.

TABLE IV
Identification of clinical isolates of yeasts by disk-diffusion test

Species	Number of isolates of stated species in		
	Series A (623)	Series B (429)	All (%) (1052)
<i>C. albicans</i>	430	302	732 (69.6)
<i>C. tropicalis</i>	59	58	117 (11.0)
<i>T. glabrata</i>	57	44	101 (9.6)
<i>C. stellatoidea</i>	15	8	23 (2.2)
<i>C. guilliermondi</i>	13	4	17 (1.6)
<i>C. parapsilosis</i>	10	6	16 (1.5)
<i>C. krusei</i>	13	0	13 (1.2)
<i>C. pseudotropicalis</i>	9	1	10 (1.0)
<i>T. candida</i>	4	2	6 (0.6)
<i>C. lipolytica</i>	3	1	4 (0.4)
<i>Geotrichum</i> sp.	2	1	3 (0.3)
<i>R. rubra</i>	2	0	2 (0.2)
Not identified	6	2	8 (0.8)

that 90.2% of the 1052 clinical isolates were identified as *C. albicans*, *C. tropicalis* or *T. glabrata*.

Profile overlap

When the species described in tables I and III were listed by codes (including the nine codes found only among isolates of series B and, therefore, not in tables I and III) in order of increasing sensitivity, it was noted that some codes represented two or more species (table V). Nevertheless, in these cases, species represented by a common code were readily differentiated when other properties, including the appearance and characteristic diameters of the zones of inhibition, were considered.

Cr. albidus and *Cr. laurenti* occur rarely in clinical material and were not represented among the 1052 clinical isolates examined in this study. Thus, *T. glabrata* is the species most likely to be associated with code 020050 in clinical practice. Furthermore, of the pairs of strains represented by the codes 000450, 020400, 103406,

TABLE V
Identification codes for yeasts by disk-diffusion test

Code	Species	Mean diameter (mm) of inhibitory zones with						Remarks
		JG	EB	TTC	BG	CY	R6G	
000000	<i>Geotrichum</i> sp.	0	0	0	0	0	0	
000006	<i>C. albicans</i>	0	0	0	0	0	15	
000050	<i>T. glabrata</i>	0	0	0	0	30	0	*TTC-48
000406	<i>C. albicans</i>	0	0	0	12	0	13.5	
000450	<i>C. parapsilosis</i>	0	0	0	15.8	19.7	0	†TTC
	<i>C. rugosa</i>	0	0	0	13	40	0	
003050	<i>T. glabrata</i>	0	0	38R	0	30.8	0	*TTC-48
003450	<i>T. glabrata</i>	0	0	36R	9.1	29.7	0	*TTC-48
020050	<i>T. glabrata</i>	0	16R	0	0	30	0	*TTC-48
	<i>Cr. laurenti</i>	0	15	0	0	30	0	Capsule
020400	<i>C. lipolytica</i>	0	19.7	0	12.7	0	0	
	<i>Geotrichum</i> sp.	0	10	0	14	0	0	Arthrospores
020406	<i>T. candida</i>	0	14.5	0	15	0	17	
020450	<i>C. parapsilosis</i>	0	10.3	0	15.3	20	0	†TTC
020456	<i>T. candida</i>	0	14.5	0	15	14	17	
023050	<i>T. glabrata</i>	0	14R	36.2R	0	30.5	0	*TTC-48
023450	<i>T. glabrata</i>	0	14.9R	36.2R	8.2	32.4	0	*TTC-48
023456	<i>C. krusei</i>	0	15R	10.3	11	31.7	10.7	Rough colonies
100006	<i>C. albicans</i>	9	0	0	0	0	15	
100406	<i>C. albicans</i>	9.5	0	0	13	0	14.5	
103006	<i>C. albicans</i>	9	0	±	0	0	15	
103406	<i>C. albicans</i>	9.5	0	±	13	0	14.5	
	<i>C. stellatoidea</i>	15	0	29.3	15	0	17	
120406	<i>C. albicans</i>	10.7	10.8	0	13.6	0	15	Germ tube
	<i>R. rubra</i>	9	17	0	20	0	17	
120450	<i>C. parapsilosis</i>	9.5	12.6	0	15.6	21.5	0	†TTC
	<i>Cr. neoformans</i>	13	18	0	24	50	0	Capsule
120456	<i>C. guilliermondi</i>	12.8	13.2	0	14.1	12.3	20.2	
123050	<i>T. glabrata</i>	12.4	18.6R	38.4R	0	27.4	0	*TTC-48
123056	<i>C. tropicalis</i>	15.8	17.4	28	0	15.6	13.6	*TTC-24
123406	<i>C. pseudotropicalis</i>	16.7	17.5	20.5	14.3	0	16.7	
	<i>T. glabrata</i>	10.9	14.3R	38.4R	10.2	32	0	*TTC-48
123450	<i>C. parapsilosis</i>	9.5	12.6	±	15.6	21.5	0	†TTC
	<i>S. cerevisiae</i>	13	15	24	12	50	0	
	<i>Cr. albidus</i>	9	25	23	20	46	0	Capsule
123456	<i>C. tropicalis</i>	15.8	17.4	28	11.8	15.6	13.6	*TTC-24
	<i>C. krusei</i>	12.8	14.4R	15.1	12	41.8	14.8	Rough colonies

* Brown ring around the TTC-disk after incubation for 24 or 48 h.

† Brown surface around the TTC-disk.

± = A very small inhibitory zone.

R = Regrowth within the inhibitory zone.

FIG. 3.—Disk-diffusion test with *C. tropicalis* (inoculated by flooding). Disks applied in same order as in fig. 1.

120406 and 120450, the greater sensitivity of one species of the pair to one or more chemicals (e.g. CY, EB, TTC, EB or BG and CY, respectively) allowed a likely identification to be made (table V). For the four species represented by code 123450, *Cr. albidus* is an unlikely identification (*vide supra*). The characteristic sensitivities of the other three species in that group to chemicals TTC and CY enable species to be identified. In the group represented by code 123456, *C. tropicalis* may be easily distinguished from *C. krusei* with chemicals TTC and CY; *C. tropicalis* also produced a typical brown-red colouration at the edge of the TTC zone (fig. 3).

DISCUSSION

Disks containing chemicals or antibiotics have long been used for the routine detection of biochemical and antibiotic reactions of bacteria. For example, identification of gram-negative anaerobic bacilli by antibiotic sensitivities alone has been described (Finegold *et al.*, 1967; Essers, 1982). The results presented here reveal that the disk-diffusion test identified all but a few (0·8%) of 1052 clinical isolates of yeasts. There was good agreement between the results obtained by this method and those from

the commercial kit (API 20C Aux) or conventional methods (Lodder and Kreger-van Rij, 1967). The agreement in identification between the disk-diffusion method and the API system was high (95.3%).

The method described is technically straightforward and gives reliable results in only 24 h for most species. Because the reactions to the chosen chemicals may be tested with six impregnated disks on only one plate, the method is inexpensive. Furthermore, the disks and the stock solutions remain stable for up to one year and the results are not subject to minor technical variations in the application of the system. The inoculum used to seed the Sabouraud agar is not critical; reproducible results were obtained even over a wide inoculum range ($c. 5 \times 10^3$ – 10^6 cells). Similarly, the method of seeding the agar did not influence the results. Regardless of inoculum size, the zones of inhibition obtained for each species showed no more than slight deviation, indicating that the interaction between the yeasts and the dyes was essentially constant, a characteristic of the system that simplified evaluation. Discrepancies were, however, observed with some makes of Sabouraud agar (e.g., Merck) for reasons that are not yet understood. Therefore, at present the disk-diffusion method may be performed on Oxoid Sabouraud agar only.

In the minority of cases in which one code identified two or more species, the species were readily identified by microscopy, colonial morphology, the characteristic appearance of the zone of inhibition and the different degrees of inhibition of each species by different chemicals. Even isolates of *T. glabrata*, which gave most profiles, were readily identified by their resistance to rhodamine 6G and by the appearance of the zones around triphenyltetrazolium chloride.

The greatest difficulty was experienced with *C. stellatoidea*. The disk-diffusion test identified 15 isolates as *C. stellatoidea* whereas the auxanogram method identified only three of these same isolates as *C. stellatoidea*, eight as *C. albicans* and four were not identified. However, seven and 12 of these isolates were putatively identified as *C. stellatoidea* by, respectively, their behaviour in conventional sucrose-assimilation tests and their formation of star-shaped colonies on blood agar after 24 h. It is of interest that most of these strains of *C. stellatoidea* had been isolated during a relatively short period from the same hospital and may have resulted from nosocomial spread.

The disk-diffusion method described offers, therefore, a reliable means for the identification of yeasts from clinical specimens. Of randomly isolated yeasts sent to our laboratory, 90% were identified at the first attempt by the combination of the disk-diffusion test and the few additional criteria detailed (table V; figs. 1–3). The potential of this simple test is high, especially because it can be applied in small laboratories without the need for extensive or expensive facilities. It is also clear, however, that further detailed examination, including tests with larger numbers of named reference strains, is required for some species, such as *C. stellatoidea* and *T. glabrata*. In future studies, the behaviour of species such as *Trichosporon capitatum*, *T. cutaneum* and *R. glutinis* in the disk diffusion test should be examined.

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